Biological characterization of South African bacteriophages infective against *Streptococcus uberis*, a causal agent of bovine mastitis

by

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DISSERTATION SUMMARY

Mastitis is an inflammatory disease of bovine mammary glands and is the most economically important disease affecting dairy herds in South Africa, and globally. The incidence and history of mastitis in South Africa has been extensively documented and found to be bacterial in origin. *Streptococcus uberis (S. uberis)* is the most common environmental causal agent of mastitis from clinical and subclinical samples in several countries, including Australia, the United Kingdom, New Zealand, and Belgium. Due to the causal agents ability to exist in cells, it remains protected from antibiotics. The quest for antibiotic free dairy management has funded the research into integrated strategies, predominantly proactive udder health management. Biological control methods are not widely used, specifically the use of bacteriophage (phage) therapy. Phages are ubiquitous (found in all biospheres) and are the most abundant organisms on earth. Understanding the interaction between phages and their hosts is vital to their manipulation for therapeutic conditions.

This study aimed to isolate phages from unpasteurized milk of dairy cows, sequentially screened these against *S. uberis* isolates demonstrating antimicrobial resistance at the time. The phages have been screened for robust lytic characteristics for the of intent of a phage-based therapy.

Bacterial strains of *S. uberis* were isolated from unpasteurized milk by Allerton laboratories submitted by dairy farms in KwaZulu-Natal, South Africa. The samples were screened for *S. uberis* using the following tests: haemolysis patterns on blood agar, catalase reactions using hydrogen peroxide (5%), and Gram reaction. The identity of the strains was then confirmed by Inqaba Biotechnical Industries (Pty) Ltd via sequencing of the 16s ribosomal RNA.

The six *S. uberis* strains were screened against 8 commonly used antibiotics in the dairy industry: β-lactam (ampicillin, penicillin G, cefalexin, oxacillin and amoxicillin), macrolides (erythromycin), tetracyclines (tetracycline) and glycopeptide (vancomycin). Using the Kirby Bauer method and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breaking points zone diameters, version 10.0, 2020. Every strain of *S. uberis* was susceptible to vancomycin, which is not available for intra-mammary infections. Resistance to the 7 other antibiotics varied amongst the

strains. This was not a survey study but an isolation of *S. uberis* strains to challenge the efficacy of phage therapy.

Approximately 2,000 mastitic milk samples were screened for phages resulting in the isolation of 95 phages. This was further reduced by screening for constant lytic ability to five phages that were characterized for their phage titre, host range, single step growth phase and lethal dose activity. The titre of the five selected phages varied considerably. Phages CP1, CP2, CP76, CP79 and CP80 produced titres of $59x10^2$, $56x10^5$, $47x10^6$, $50x10^4$ and $35x10^4$ pfu.ml^{-1}, respectively. There was a differential interaction between the five phages isolates and the six strains of *S. uberis*. One strain of *S. uberis* was susceptible to all five phages, Strain 21A, whereas Strains 17D and 78B were not susceptible to any of the five phages. Phage CP2 was virulent to four of the six *S. uberis* strains. The single step growth assay illustrated the cyclic replication of the isolated phages takes between 50 - 60 minutes. In a lethal dose assay, Phages CP1 and CP2 were each able to reduce *S. uberis* counts by 86% and 83%, respectively. Of the 5 phages screened Phages CP1 and CP2 showed potential as stand-alone treatments, whereas Phages CP76, CP79 and CP80 would offer better control when combined in a phage cocktail, and this would broaden the host range. Phage samples were examined using transmission electron microscopy (JEOL 1400). Various negative stains were used to view the virus particles: 2% uranyl acetate (UA), 2% phosphotungstic acid (PTA) and 0.05-5% ammonium molybdate (AM). The AM staining provided the best images of infected bacterial cells and phage surface structures. All micrographs obtained, illustrated similar viral particle structures suggesting the phages screened belong to one family. A virus particle was measured to have a 50-65nm diameter icosahedral head and a short tail ranging from 25-35nm in length from the EM micrograph. The virus particles exhibited *Podoviridae* morphology. However, only a complete genomic sequencing will confirm the identity of these virus particles to a species level.

DECLARATION

I, Caleb Pillay, declare that:

(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

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DISSERTATION INTRODUCTION

Bovine mastitis is caused by numerous pathogens including mycoplasma, viruses, yeasts and algae with the most prominent being bacteria (Batavani *et al*., 2007). The etiological agents of mastitis vary amongst countries, depending on the climate, animal species and husbandry practices (Watts, 1988; Bradley, 2002). In most cases, the causal agent of mastitis in Africa are of bacterial origin (Bradley, 2002).

Apart from reducing the milk yield by up to 30%, mastitis has a detrimental effect on milk composition and its physicochemical constitution (Batavani *et al*., 2007). The change is a result of the mammary gland being infected, resulting in inflammation, and abnormal cell and enzyme activity (Walstra *et al.*, 2006). Mastitis also changes the concentration and quality of protein that would normally be present in a healthy cow (Ogola *et al*., 2007). Furthermore, undesirable enzymes that are produced in excess during infection reduces the quality of milk and its production value (Ogola *et al*., 2007). The accumulation of detrimental traits lowers the value of milk to the point where it becomes unmarketable, thereby incurring losses to the farmer (Bradley, 2002; Batavani *et al*., 2007).

Previously antibiotics were not only used in the presence of a bacterial infection but as a preventative measure during the drying off period (Blowey and Edmondson, 2010). The antibiotics tetracycline and streptomycin are commonly administered to herds during the dry periods as a precautionary step (Barkema *et al*., 2006; Call *et al*., 2008; Oliver *et al*., 2011). The continuous use of antibiotics as a preventative measure has previously been documented by Oliver *et al*. (2011), to be a causal agent of the emergence of bacterial drug resistance in mastitic pathogens. However, Karzis *et al*. (2018) reported no increase of bacterial resistance in the South African dairy industry for the last 10 years.

The long-term and frequent use of antibiotics as the sole control measure used to manage mastitis inevitably leads to antibiotic resistance on dairy farms. It cannot be seen as abuse or misuse, given that antibiotic use is strictly controlled by veterinarians depending on the country's regulatory laws, it is simply bacterial evolution in action. Although bacterial resistance is not a current problem in the South African dairy

industry. There is great urgency to develop alternative therapies to alleviate the diminishing efficacy of antibiotics worldwide reported by Vanderhaeghen *et al*. (2010). The widespread development of multidrug-resistant bacteria such as *Pseudomonas* spp. (Ahmad *et al*., 2002), *Klebsiella pneumonia* (Vinodkumar *et al*., 2005), *Escherichia coli* (Blowey and Edmondson, 2010), vancomycin-resistant *Enterococci* (Biswas *et al*., 2002) and *Staphylococci* (Blowey and Edmondson, 2010), and methicillin-resistant *Staphylococcus aureus* (Basdew and Laing, 2011), has led the Western countries to renew their research programmes into phage therapy.

Phages are naturally occurring viruses that are a part of the microbial ecosystem, with the natural environment containing billions of phages (Chanishvili *et al*., 2001; Garcia *et al*., 2009). Phages have specific bacterial hosts and can infect bacteria without harming the plant or animal host of the bacterium (Fischetti, 2005).

In the global food industry, phages are being developed as alternatives to disinfectants (Sofos, 2008). Since 2006, phage applications have been approved by legislation for the elimination of *Listeria monocytogenes* (Sofos, 2008).

The objective of this project was to isolate and characterise phages capable of lysis of *in vitro* bovine *S. uberis*. The research was envisaged as a precursor to *in vivo* trials on dairy herds, to support or replace antibiotic therapy. The specific research objectives of the current study were therefore as follows:

1. Review of literature on the impact of bovine mastitis on the dairy industry, the nature of the mastitis infection and the cow's defence mechanisms, the history and evolution of antibiotic resistance in the dairy industry and the potential of phage therapy in dairy production.
2. Identify resistant *S*. *uberis* strains present in KwaZulu-Natal (KZN) to access the phage therapy efficacy in chapter 3.
3. Isolate, screen and characterize *S. uberis* phages in the following categories: phage titre, host range, single step growth phase and lethal dose activity.
4. Morphologically characterise the *S. uberis* phages using a transmission electron microscopy (JEOL 1400).

This dissertation comprises five chapters; one is a literature review, three are research chapters on the isolation of *S*. *uberis* strains and phages, following the

characterization of antibiotic resistance and phage lytic ability. The final chapter is an overview of the research covered in the dissertation, identifying key findings and future research. Each chapter is written in the form of a scientific paper. As a result, there is some unavoidable repetition of prior information and references. However, this is the recommend format adopted by the University of KwaZulu-Natal for theses. The references are formatted in accordance with the Journal of General Virology.

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CHAPTER 1

Review of Literature

1.1 Introduction

Bovine mastitis is an inflammation of the parenchyma of mammary glands of dairy cows (Jones and Bailey, 2009). The symptoms comprise of physical and chemical changes of the glandular tissue and of milk, adversely affecting the cow's health and quality of milk respectively (Blowey and Edmondson, 2010). The variety of pathogens that can infect a cow's udder was demonstrated by Watts, who isolated and identified 137 different pathogens from mastitis-infected cows (Watts, 1988). Contamination may originate from numerous sources in the milking process and housing of the cow, including contaminated milking equipment, staff and unsanitary stalls (Watts, 1988; Bradley, 2002). The etiological agents of mastitis vary between countries, depending on the climate, animal species and husbandry practices (Watts, 1988; Bradley, 2002). The ubiquity of these pathogens, coupled with the endemic nature of the disease, makes complete eradication impossible (Blowey and Edmondson, 2010). Mastitis is recognized as a disease complex, which is a disease involving various factors. Identifying the main pathogens and risk factors are fundamental to developing preventive and control measures (Harding, 1995; Van den Borne, 2010). The common causal agents of mastitis in Africa are of bacterial origin, with five common species: *Escherichia coli*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus agalactia* and *Staphylococcus aureus* (Bradley, 2002).

Mastitis is the most economically important disease affecting dairy herds worldwide, with losses arising from decreased milk production, reduced milk quality, veterinary bills and culling (Batavani *et al*., 2007; Kudi *et al*., 2009). Clinical mastitis costs the UK dairy industry an excess of USD 192 million annually (Bradley, 2002). In 1976, annual losses from mastitis in the USA were estimated at a total of USD 1,294 million and these losses had increased to USD 2 billion by 2009 (Viguier, 2009). Worldwide collective mastitis losses are estimated to be USD 35 billion annually (Jones and Bailey, 2009; Van den Borne, 2010).

A mastitis infected cow is usually treated with antibiotics to eliminate the infection as the first defense dependent of the bacterial species (Reyher and Dohoo, 2011).

However, milk containing quantities of antibiotic residues exceeding the regulated limit will be dumped, causing further financial losses to the already incurred reduced milk turn-over, culling and veterinary expenses attributed to mastitis (Reyher and Dohoo, 2011). The adverse effects are not limited to financial losses. Extensive use of antibiotics in the treatment of mastitis increases the probability of creating antibiotic-resistant strains of the causal bacterium (Mangili *et al*., 2005; Shi *et al*., 2010). The inevitable development of antibiotic resistance has driven a search for alternative control measures to manage the disease (Bradley, 2002).

One such measure is the use of biological control to treat mastitis, specifically the use of phage therapy to treat pathogenic bacterial infections (Garcia *et al*., 2009; Basdew and Laing, 2011). Phage therapy targets a specific strain of bacterium and is harmless to the host and beneficial bacterium (Basdew and Laing, 2011), unlike antibiotics which have a wider target spectrum, other than the target pathogen (Chanishvili *et al*., 2001). With the pressure of antibiotic resistance looming, the concept of phage therapy has grown in popularity (Chanishvili *et al*., 2001). Many phage applications are showing promise and have been approved against food pathogens (Hagens and Offerhaus, 2008). An example is Listex, a commercial phage therapy used in the United States against *Listeria monocytogenes,* a common food poisoning pathogen (Hagens and Offerhaus, 2008). Phage therapy is also regularly administered in Georgia, Poland and Russia on human pathogens that do not respond to antibiotic treatments (Fischetti, 2005). However, the development of phage therapy to manage mastitic bacteria has not reached the stage of registration yet (Garcia *et al*., 2009).

This review covers the current developments in bovine mastitis, with a focus on the *Streptococcus* species and phage therapy as a preventative treatment.

1.2 Mastitis

Mastitis is a complex disease due to the number of etiological agents responsible for the disease. Hence, identifying the main causal agents is essential for control (Harmon, 1995; Van den Borne, 2010). Mastitis is classified into two dynamic groups, determined by the pathogen source and mode of transmission, i.e., contagious and environmental (Bradley, 2002). To date, more than 200 potentially pathogenic species of organisms have been shown to cause bovine mastitis (Watts, 1988; Petrovski *et al*., 2011). There is a large number of less common pathogens that are difficult to categorise as environmental or contagious due to their varying characteristics (Blowey and Edmondson, 2010). Contagious mastitis is further subdivided into clinical mastitis, sub-clinical mastitis and chronic mastitis (Bradley, 2002; Jones and Bailey, 2009). Clinical mastitis is characterized by systemic symptoms (change in behaviour, loss of appetite, fever), inflammation of the udder and a visual change in the composition of the milk (Kudi *et al*., 2009). Sub-clinical mastitis results in a change in milk composition without visual symptoms on the infected udder such as inflammation or milk abnormalities (Batavani *et al*., 2007). Chronic mastitis comprises a mixture of symptoms that occur periodically (Bradley, 2002).

1.2.1 Contagious Pathogens

Contagious pathogens are primarily located on the teat of the cow, which then serves as a reservoir of infection, resulting in microbes spreading from one cow to the next during milking (Kudi *et al*., 2009). Contagious pathogens include *Staphylococcus aureus, Streptococcus agalactia*, *Mycoplasma* spp. and *Corynebacterium bovis* (Bradley, 2002; Jones and Bailey, 2009). There is a large number of less common pathogens that are difficult to categorise as environmental or contagious due to their varying characteristics (Blowey and Edmondson, 2010).

1.2.2 Environmental Pathogens

Environmental pathogens are pathogens that do not ordinarily live on the animal; but are introduced onto the teat of the cow from the environment (Blowey and Edmondson,

2010). These pathogens may be transferred from the bedding material, faecal matter, feed or workers (Kudi *et al*., 2009). The most frequently isolated environmental pathogens are usually *Staphylococcus uberis*, *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp, (Bradley, 2002; Kudi *et al*., 2009; Blowey and Edmondson, 2010). The incidence of these pathogens fluctuates with time and correlates with a decrease in *Staphylococcus aureus* diagnoses (Blowey and Edmondson, 2010).

1.3 Prominent Etiological Agents of Mastitis

1.3.1 *Staphylococcus aureus*

This bacterium is Gram-positive, coccus-shaped, haemolytic and a facultative anaerobe (Madigan *et al.*, 2012). *S. aureus* was the most prominent etiological agent of clinical and subclinical mastitis cases found in dairy herds globally and is classified as a contagious pathogen (Workineh *et al*., 2002; Barrett *et al*., 2005; Jones and Bailey, 2009; Basdew and Laing, 2011). Recent studies have reported 10% of mastitis cases have been linked *S*. *aureus* (Karzis *et al*., 2018).

The development of antibiotic resistance in *S*. *aureus* has been well documented and makes the control of mastitis outbreaks difficult and ineffective (Basdew and Laing, 2011). Many dairy farmers have implemented mastitis prevention programmes to avoid *S*. *aureus* outbreaks (Bradley, 2002; Barkema *et al*., 2009). The reason for the poor response to antibiotic therapy is due to the biological functions of the bacterium (Bradley, 2002). Antibiotics circulate within the bodily fluids but are unable to penetrate cells (Barkema *et al*., 2009). In contrast, *S*. *aureus* can penetrate and live within macrophages, out of reach of antibiotics (Barkema *et al*., 2009). Once an *S*. *aureus* infection is established within the mammary tissue, it forms a fibrous wall around itself that is almost impermeable to antibiotics (Workineh *et al*., 2002). These two characteristics result in chronic, recurring episodes of mastitis in specific cows, which show limited responsiveness to treatment (Workineh *et al*., 2002; Barkema *et al*., 2009). Methicillin-resistant *S. aureus* (MRSA) is one of the popular public health concerns associated with bovine mastitis (Workineh *et al*., 2002; Barkema *et al*., 2009). Due to the zoonotic transmission possibility, MRSA poses a great risk to the human population (Barkema *et al*., 2009).

1.3.2 *Streptococcus agalactiae*

Streptococcus agalactiae is a Gram-positive, coccus-shaped, β-haemolytic bacterium (Madigan *et al*., 2012). It is classified as a contagious pathogen because the primary source of infection is within the mammary gland, the teat canal and the teat skin (Barkema *et al*., 2009). Developed countries have been able to eliminate *S. agalactiae* from dairy herds by adhering to recommended control strategies (Andersen *et al*., 2003; Olde Riekerink *et al*., 2006). It is responsive to antibiotic therapy although being highly contagious, resulting in the marked success rate of elimination from dairy herds (Olde Riekerink *et al*., 2006). If *S. agalactiae* is present in a dairy herd, it is a general indicator of poor hygiene during the milking process (Andersen *et al*., 2003).

1.3.3 *Streptococcus dysgalactiae*

Streptococcus dysgalactiae is a Gram-positive, coccus-shaped, β-haemolytic bacterium (Madigan *et al.* 2012). It is both environmental and contagious as the pathogen can be transmitted during milking and can be found on organic matter in the general vicinity of the animal (Barkema *et al*., 2009). Successful treatment of *S. dysgalactiae* is common with the use of broad-spectrum intermammary antibiotics (Nickerson, 2009). Sanitary precautions and proper milking procedures increase the probability of prevention of this pathogen (Nickerson, 2009).

1.3.4 *Escherichia coli*

Escherichia coli causes both a non-persistent and persistent intermammary infection (Blowey and Edmondson, 2010). In non-persistent cases, the infection undergoes self-cure (Blowey and Edmondson, 2010). The symptoms exhibited are largely dependent on the host characteristics. Predominantly, the symptoms are a hard, hot, swollen quarter, with a watery discharge (Burvenich *et al*., 2003). Cases of cows dying from a shock reaction due to an *E. coli* infection is a result of an endotoxin (Burvenich *et al*., 2003). Responses to antibiotic treatment of this bacterium varies, depending on the type strain present, due to its genetic diversity (Burvenich *et al*., 2003). Some strains

have demonstrated increased resistance to some antibiotic groups: tetracycline, β-lactam agents and lincomycin (Burvenich *et al*., 2003).

1.3.5 *Streptococcus uberis*

Streptococcus uberis is a Gram-positive, coccus-shaped, non-haemolytic bacterium that causes environmental bovine mastitis (Madigan *et al*., 2012). This bacterium is capable of both clinical and subclinical infections of cows (Almeida *et al*., 2006). "*S. uberis* has been repeatedly identified as the most commonly isolated pathogen from clinical and subclinical samples in several countries, including Australia, the United Kingdom, New Zealand, and Belgium", (Davies *et al*., 2016). *S. uberis* has been documented to show little responsiveness to antibiotic treatments (Burvenich *et al*., 2003; Blowey and Edmondson, 2010). Compounded with its ability to move into the lymph nodes, it is a persistent nuisance to the dairy community (Almeida *et al*., 2006; Nickerson, 2009). The pathogen is extremely versatile and is able to survive in various areas of cows: mouth, vulva, groin and axilla (Almeida *et al*., 2006; Blowey and Edmondson, 2010). The bacterium is identified as an environmental pathogen and is found in the bedding (straw and sand), pastures and faeces (Almeida *et al*., 2006; Blowey and Edmondson, 2010). Dairy cows are constantly exposed to *S. uberis* due to the ubiquity of the pathogen. Once cows are infected, the pathogen may behave as a contagious infection (Almeida *et al*., 2006). The symptoms include hard, swollen quarters, clotting of milk and a fever. *S. uberis* has been documented with a large strain diversity, varying in pathogenicity (Barkema *et al*., 2009). Some strains have also shown resistance to phagocytosis in the presence of the milk protein casein (Hillerton and Kliem, 2002). Originally *S. uberis* was classified an environmental pathogen of low importance (Erskine *et al*., 2002). Currently, the pathogen shows low sensitivity to antibiotic therapy, resulting in chronic and recurrent subclinical mastitis (Hillerton and Kliem, 2002). Thus, early detection of this pathogen is required to isolate infected animals to prevent further spread (Hillerton and Kliem, 2002). Due to its poor opsonization in the presence of casein protein and its ability to exist in cells, it remains protected from antibiotics (Hillerton and Kliem, 2002). *S. uberis* is the most common environmental mastitis pathogen in most countries (Barkema *et al*., 2009; Blowey and Edmondson, 2010).

1.3.6 Coagulase-Negative Staphylococci (CNS)

Coagulase-negative staphylococci (CNS) are Gram-positive, coccus-shaped bacteria that can either be haemolytic or non-haemolytic, and are coagulase negative (Erskine *et al*., 2002). More than 50 species have been classified as CNS and can cause both clinical and subclinical mastitis (Pyörälä and Taponen, 2009; Thorberg, *et al*., 2009). The predominate CNS species identified causing intra-mammary infections were *S. chromogenes*, *S. haemolyticus*, *S. simulans* and *S. epidermidis*. Other CNS species identified at lower frequencies included *S. hominis*, *S. auricularis*, *S. sciuri*, *S. capitis*, *S. cohnii*, *S. warneri*, *S. pasteuri, S. xylosus, S. hyicus, S. equorum, S. microti, S. rostri, S. gallinarum, S. saprophyticus* and *S. succinus* (Jenkins *et al*., 2019; Taponen *et al*., 2008; Pyörälä; Taponen, 2009). In Belgium, *S. chromogenes* was documented to be the predominate CNS species (Jenkins *et al*., 2019). The occurrence of CNS in US and European dairy herds has been documented to infect up to 55% of the heard (Jenkins *et al*., 2019). Petzer *et al*., (2016) reported an isolation of CNS pathogens from 12.4% of quarters with high SCC in South African commercial dairy herds. They are commonly found in the environment, teat skin, teat ends, nasal passages and milker's hands (Thorberg *et al*., 2009). Previously CNS infections underwent self-cure, currently data shows an increased trend of occurrence rates and persistent infections (Jenkins *et al*., 2019; Pyörälä and Taponen, 2009).

1.4 Effect of Mastitis on Milk Composition

Apart from reducing the milk yield by up to 30%, mastitis has a detrimental effect on milk composition and physicochemical constitution (Batavani *et al*., 2007). The change is a result of the mammary gland being infected, resulting in inflammation and abnormal cell and enzyme activity (Walstra *et al.*, 2006). Internal swelling usually follows, and the mammary gland alveoli become structurally damaged (Schukken *et al*., 2004). The blood-milk barrier then becomes compromised, which leads to the release of unregulated chloride, sodium, hydrogen, potassium and hydroxide ions into the milk, thereby increasing the conductivity and pH of the milk (Schukken *et al*., 2004). Typical external symptoms of severe infection include swelling and reddening of the udder (Blowey and Edmondson, 2010). Milk also contains a higher water content and

visible blood and clots (Schukken *et al*., 2004). If left untreated, the infection may result in the animal's death (Blowey and Edmondson, 2010).

Lactose concentrations in milk of mastitis infected cows decrease, which results in increased sodium and chloride levels to maintain an osmotic balance (Blowey and Edmondson, 2010). These increases can be measured by the electrical conductivity (EC) of the milk because the increased concentrations of sodium and chloride ions increase the EC of the milk (Walstra *et al*., 2006). A high salt concentration imparts a salty, bitter taste to infected milk (Walstra *et al.*, 2006; Blowey and Edmondson, 2010). Mastitis also changes the concentration and quality of protein present in milk. (Ogola *et al*., 2007). Casein is the primary protein present in milk that is required for cheese and yoghurt production (Ogola *et al*., 2007). A low concentration of casein affects the flavour and quality of cheese and yoghurt (Harmon, 1995; Ogola *et al*., 2007). Furthermore, undesirable enzymes that are produced in excess during infection reduce the quality of milk and its production value (Ogola *et al*., 2007). The enzyme plasmin, which degrades casein, has been found at higher levels after mastitis infection (Harmon, 1995). The enzyme is not denatured during pasteurization and is active during cold storage, reducing shelf life (Harmon, 1995). The enzyme lipase degrades fat in milk into fatty acids, which hinders starter cultures in cheese and milk production (Harding, 1995). Increased concentrations of free fatty acids also leads to a rancid flavour in milk (Harding, 1995). The accumulation of undesirable properties in contaminated milk caused by mastitis lowers the value of milk to the extent where it becomes unmarketable, thereby incurring direct financial losses to the farmer (Bradley, 2002; Batavani *et al*., 2007).

1.5 Transmission of Mastitis

The detailed epidemiology of mastitis will allow for further insight into the development of adequate detection and control techniques. The presence of lactose and the warm temperature of bovine mammary glands promotes rapid growth of Gram-negative, lactose-fermenting pathogens (Workineh *et al*., 2002; Barkema *et al*., 2009). The pathogen enters the teat of a cow from a reservoir that is either from the environment or introduced from another cow (Barkema *et al*., 2009). In the environment, these

pathogens are ever-present. However, it does not become problematic until environmental conditions favour the rapid population rise of one micro-organism (Madigan *et al.* 2012). Frequently encountered environmental pathogens that cause bovine mastitis are *E*. *coli*, *S*. *uberis*, *Bacillus* spp., fungi and yeasts which is variable depending on the climate, changing environmental conditions and farming strategies (Barkema *et al*., 2009; Madigan *et al.,* 2012).

The spread of pathogens between cows is generally through contaminated equipment or staff members, and these pathogens and are referred to as contagious (Blowey and Edmondson, 2010). Typical examples include *S*. *aureus*, *S*. *agalactiae* and *S*. *dysgalactiae* (Blowey and Edmondson, 2010). The contagious pathogens can also be introduced from new cows that are added to the herd (Barkema *et al*., 2009).

The pathogen enters via the teat canal, which is naturally closed by a sphincter muscle as a defence response to pathogens (Dogan *et al*., 2006). However, after milking, the teat canal may remain dilated open for up to 120 minutes, and if damaged, the teat canal may remain partially open indefinitely, allowing pathogens to colonize the teat canal and mammary tissue (Barkema *et al*., 2009). Milking equipment may also propel the pathogen into the teat by a reverse flow of milk (Schukken *et al*., 2004; Dogan *et al*., 2006). If the pathogen can avoid the natural defence mechanisms (cellular and humoral) of the cow, it will establish itself inside the udder and will replicate exponentially (Schukken *et al*., 2004; Barkema *et al*., 2009).

Research has shown that a dairy cow is increasingly vulnerable to intramammary infections during a number of physiological transitions including: as calving approaches, early non-lactating periods (drying off) and the early lactation periods (Nickerson, 2009). Understanding the periods of high susceptibility to infection and the life cycles of the causal agents has allowed for the appropriate preventative protocols to be established, thereby lowering disease incidence and ensuring stable production of high-quality milk (Nickerson, 2009).

1.6 Teat and Udder Defense Mechanisms

A cow's mammary gland defence system employs a physical barrier and physiological mechanisms to alert the immune system of foreign bodies (Blowey and Edmondson, 2010). The immune response is a multifaceted system comprising of a cellular response using leukocytes (granulocytes, lymphocytes and monocytes), and a hormonal response using soluble immune components such as antibodies and enzymes (Walstra *et al.* 2006; Blowey and Edmondson, 2010).

1.6.1 Teat Defences

The teat canal connects the teat orifice to the teat cistern which is a passageway that bacterial cells need to colonize or travel along in order to establish an infection (Fig. 1.1) (Walstra *et al.* 2006; Blowey and Edmondson, 2010). The teat canal is lined with stratified squamous epithelium, (dead skin cells filled with keratin), which forms an impermeable physical barrier to bacterial cells, thus preventing their growth (Tyler and Ensminger, 1993). The entrance to the teat is sealed by a sphincter valve (Fig. 1.1), physically stopping the entry of foreign bodies (Juozaitiene *et al*., 2006). The teat canal ranges from 5-13 mm long and is lined with a bacteriostatic keratinised skin epidermis containing lipid keratin and cationic proteins (Seykora and McDaniel, 1985; Juozaitiene *et al*., 2006). The keratin fatty acids are bacteriostatic, with cationic proteins attached to them (Tyler and Ensminger, 1993). The proteins bind and break down Gram-positive bacterial cell walls, killing the bacteria (Tyler and Ensminger, 1993). A forced purge of the teat canal occurs post-milking to push out any bacterial cells that may have entered because the canal remains dilated during milking (Seykora and McDaniel, 1985). Specialised cells in the teat canal called the Rosette of Furstenberg, release lymphocytes as a precautionary function after milking (Juozaitiene *et al*., 2006). After the lactation period, teats secret a waxy keratin plug which seals the teat end and acts as a physical barrier assisting the sphincter muscle, but this eventually dissolves as calving approaches (Seykora and McDaniel, 1985; Tyler and Ensminger, 1993).

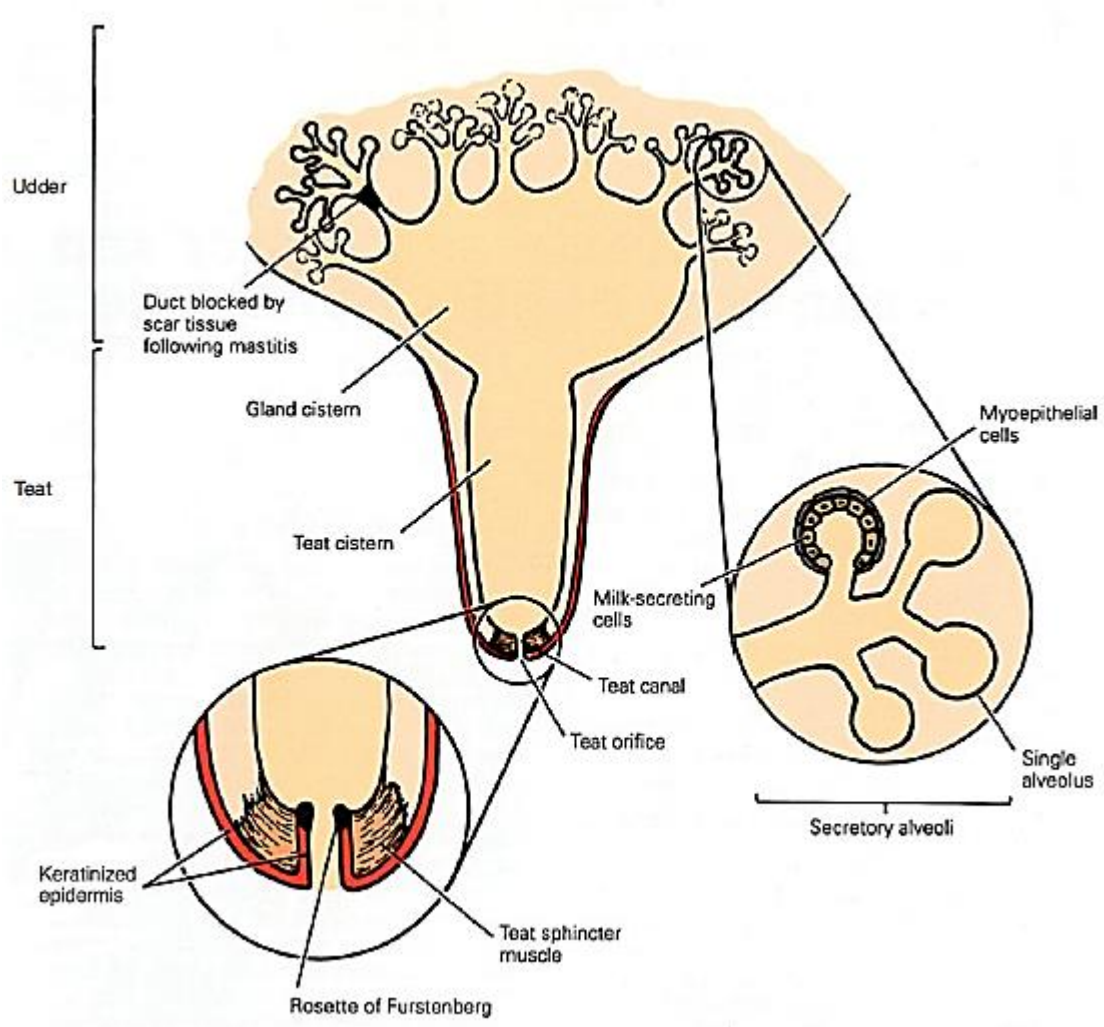

Figure 1.1 The bovine udder and teat structure (Blowey and Edmondson, 2010).

1.6.2 Udder Defences

Infectious bacteria and fungi may circumvent the teat physical barriers and establish colonies in the mammary tissue (Walstra *et al.* 2006). This may occur by colonization of the teat canal after milking, or by direct inoculation via propulsion into the teat through reverse flow during milking (Blowey and Edmondson, 2010). Once a bacterial infection is established in the mammary gland, it will trigger a dual phase defence mechanism. The immune response occurs as an intrinsic defence that is continuously present as a precautionary mechanism, and secondly, as an inducible response that occurs when an infection is detected (Walstra *et al.* 2006).

1.6.2.1 Intrinsic Defence

The intrinsic defence system utilizes a multifaceted array of enzymes, antibodies and cellular responses to initiate defence (Walstra *et al.* 2006).

Lactoferrin is an enzyme that binds to iron in udder secretions, reducing the availability of it to bind with citrate and become biological available to pathogenic bacteria (Leitner *et al*., 2000). The effects of lactoferrin are reduced during lactation but are mostly active during the non-lactating periods (Leitner *et al*., 2000; Walstra *et al.* 2006).

Lactoperoxidase is an enzyme impedes the growth of Gram-positive bacteria and kills Gram-negative bacterium (Leitner *et al*., 2000; Walstra *et al.,* 2006). Lactoperoxidase works in direct conjunction with thiocyanate and hydrogen peroxide, i.e., the higher the levels of thiocyanate and hydrogen peroxide, the more effective lactoperoxidase is (Blowey and Edmondson, 2010).

Antibodies are present in high levels in the colostrum, and function by binding to antigen receptors on the bacterium (Blowey and Edmondson, 2010). This binding process triggers a response from white blood cells to engulf the identified bacterium increasing the efficacy of this immune reaction (Petzl *et al*., 2008).

Somatic cells are present in the mammary tissue, and function by killing bacteria, thereby preventing infection (Sutra and Poutrel, 1994). Typical somatic cells induce neutrophils, vacuolated macrophages, non-vacuolated macrophages, lymphocytes and duct cells. A total cell count of somatic cells is termed the somatic cell count (SCC) (Sutra and Poutrel, 1994; Petzl *et al*., 2008). These cells vary in concentration, depending on the stage of lactation and the presence pathogens. They function as triggers of the immune system upon recognition of an infection, leading to an induced immune response (Petzl *et al*., 2008; Blowey and Edmondson, 2010).

1.6.2.2 Induced Defence

The induced defence is a response to the presence of an infection that has overcome the intrinsic defence (Leitner *et al*., 2000). This response elicits multiple immune reactions to curb the infection (Leitner *et al*., 2000; Blowey and Edmondson, 2010).

The chemotaxin alarm is the signal triggered by the detection of metabolic by-products of bacterial replication within the udder (Petzl *et al*., 2008). By-products released during phagocytosis of the bacterium by macrophages, and polymorphonuclear leucocytes, are also known to trigger an immune response (Petzl *et al*., 2008).

The inflammatory response is the immune reaction signalled by the chemotaxin alarm (Blowey and Edmondson, 2010). It redirects the polymorphonuclear leucocytes present in blood vessels in the teat to the cistern and ducts of the mammary tissue (Juozaitiene *et al*., 2006; Petzl *et al*., 2008). The quarter that triggered the inflammatory response will become swollen and painful due to the increased blood flow (Petzl *et al*., 2008). Blood vessels increase in diameter, increasing the blood supply and the numbers of polymorphonuclear leucocytes that are delivered to the infection site (Walstra *et al.* 2006). Polymorphonuclear leucocytes move through the capillaries, across the mammary tissue and into the site of infection to engulf the bacteria (Walstra *et al.* 2006). The increase in the number of cells in milk due to the inflammatory response is exponential from a base level (Blowey and Edmondson, 2010). The induced response, including inflammation and a high SCC, will persist for a short period after the bacteria have been destroyed (Walstra *et al.* 2006).

1.7 Antibiotic Treatment of Dairy Herds

The lack of data correlating antibiotic resistance development and increased susceptibility to mastitis is another obstacle to the development of alternative treatment therapies in South Africa. Whilst the zoonotic potential of the bovine mastitis pathogens has not been clearly documented, it is a cause for growing concern as more species develop antibiotic resistance (Oliver *et al*., 2011). *Methicillin-resistant S. aureus* (MRSA) is a widely spread pathogen of humans and cows. MRSA contains the gene *mecA* which confers high levels of resistance to β-lactams (Vanderhaeghen *et*

al., 2010). Cases of MRSA associated with bovine mastitis have been documented in: Switzerland, Korea, Germany, Belgium, Switzerland, Japan and Turkey, where it has been found that the horizontal transfer of multi-antibiotic resistant genetic material between human and bovine strains of MRSA was possible (Vanderhaeghen *et al*., 2010). The rise of antibiotic-resistant pathogens has led to the search for viable alternatives to antibiotic therapy. Staphylococcal chromosomal cassettes (SCCs), which vary in size, usually contain additional genetic information, including genes encoding resistance to multiple classes of antimicrobials (Morgan, 2008). The smallest cassette containing a mecA gene, SCCmec type IV, is present in clones of CA-MRSA, which are becoming endemic in many parts of the world, excluding Africa (Morgan, 2008). Morgan, (2008) reported MRSA ST398 infection locations which has zoonotic risk as sporadic in livestock in the Netherlands and France (Morgan, 2008). Springer *et al*. (2009) stated, "Evidence has also identified a link between colonization of livestock and MRSA carriage and infections in people who work with animals", in Europe. MRSA as not been seen as a zoonotic threat historically, yet strain ST398 is showing a high percentage of lineage strains in Austria (Springer *et al*., 2009). The main concern would be the spread of the ST398 lineage across livestock industries via reverse zoonosis or introduced into the health care sector (Springer *et al*., 2009).

1.8 Antibiotic Resistance Screening

The discovery of the first antibiotic in the 1920s has played a pivotal role in the treatment of pathogenic diseases of mammals (Blowey and Edmondson, 2010). Since then antimicrobial chemotherapy research has yielded a plethora of antimicrobial agents currently employed for clinical use.

The Kirby Bauer disc diffusion method allows for the identification of bacterial resistance to a range of antibiotics (Bauer *et al.*, 1966). This method involves measuring the inhibition zones created by antibiotic infused discs placed onto the surface of inoculated agar. Using a reference table, the size of the inhibition zone can determine if the bacterium is susceptible (S), intermediately susceptible (I), or resistant (R) to that antibiotic (Bauer *et al.*, 1966). The European Committee on Antimicrobial

Susceptibility Testing (EUCAST) is an example of clinical breaking points zone diameters constantly being evaluated.

1.9 Alternative Therapies

A variety of alternative therapies are currently being investigated, as the world moves towards a more proactive stance of using antibiotics only when required.

1.9.1 Vaccinations

Vaccinations have been used with limited efficacy as a preventative measure against bovine mastitis (Mamo *et al*., 1994; Giraudo *et al*., 1997). It is unlikely that a multipurpose vaccine will be created to make dairy cows completely resistant to all forms of mastitis, due to the plethora of causal agents, and the constantly changing genomes of the bacteria. The goal of vaccination research is to create an immunization schedule to protect cows against a range of isolates of one pathogen because multiple strains can be present within a dairy herd and individual cows (Blowey and Edmondson, 2010). Each vaccine will need to be tailored to the specific isolates present in a particular region or country. In one study, vaccines created for *S. aureus* produced variable results, depending on the age of the cow and the environmental conditions (Giraudo *et al*., 1997). *S. aureus* vaccines derived from bacterins (attenuated bacteria) have been shown to be less protective than vaccines derived from DNA and recombinant protein (Middleton *et al*., 2009). There are mastitis-causal agents such as *Klebsiella pneumonia*, a Gram-negative rod-shaped bacterium, for which a vaccine has not been developed yet (Middleton *et al*., 2009). An example of a successful vaccine is the *Escherichia coli* J5 vaccine, which reduced the incidence of infection by 70-80% in the targeted herd (Middleton *et al*., 2009). There is a range of vaccines that are no longer effective due to strain recombination and the emergence of new strains. Hence, the need to develop new vaccines against each pathogen is a continuous process.

1.9.2 Nanoparticles

Nanotechnology has led to the development of nanosized antibacterial particles that have demonstrated elevated interactions with bacteria when compared to microparticles (Dehkordi *et al*., 2011). These particles enter cells more readily and have an increased surface area ratio (Dehkordi *et al*., 2011). The literature documents research to augment antibiotics with nanoparticles that had an increased efficacy in mastitis therapy. Dehkordi *et al*. (2011) showed that silver nanoparticles, used in combination with antibiotics, inhibited protein synthesis and had a synergistic effect against *S. aureus*. Wang *et al*. (2012) illustrated the stand-alone properties of tilmicosin-solid lipid nanoparticles as a therapy against *S. aureus*. In addition to the low cost of manufacturing this product (Dehkordi *et al*., 2011), antimicrobial agents encapsulated in nanoparticles shows promise as a commercial product for the management of bovine mastitis.

1.9.3 Cytokines

Cytokines are proteins manufactured during the signalling of an immune response (leukocyte recruitment and activation) in the presence of an infection and have been linked to stimulating acquired immunity in the mammary glands (Bradley, 2002). Literature shows that cytokines cannot trigger complete bovine immunity against mastitis. However, when administered in conjunction with antibiotics, there was a synergistic effect documented against a bovine *S. aureus* infection (Bradley, 2002).

1.9.4 Plant-Derived Antimicrobials

Plant-derived molecules and extracts have been found to have antimicrobial properties with the advantage of not inducing resistance after prolonged use (Baskaran *et al*., 2009). Baskaran *et al*. (2009) demonstrated *in-vitro*, that plant-derived molecules trans-cinnamaldehyde, eugenol, carvacrol and thymol were effective against five bovine mastitis causal agents, *S*. *agalactiae*, *S. dysgalactiae*, *S*. *uberis*, *S*. *aureus*, and *E*. *coli*. These plant-derived molecules were classified as safe by the United States

Food and Drug Administration and it was suggested that these molecules could be used as adjunctive therapy with antibiotics (Baskaran *et al*., 2009).

1.9.5 Animal-Derived Antimicrobials

Lactoferrin is an immunomodulator naturally secreted by mammals, that is found in the saliva, tears, bronchial mucus and milk (Kutila *et al*., 2003). Kutila *et al*. (2003) demonstrated the antimicrobial properties of lactoferrin against mastitis causal agents *E. coli, S. aureus*, coagulase-negative staphylococci, *Pseudomonas aeruginosa* and *K. pneumonia*. A They also showed that a combination of β-Lactoglobulin (naturally occurring in whey protein) and lactoferrin had synergistic activity against *S. aureus*. These proteins are not commercially available as antimicrobials, but further research may result in the development of animal-derived antimicrobial therapies.

1.10 Phages

Phages are naturally occurring viruses that are a part of the microbial ecosystem, with the natural environment containing billions of phages (Chanishvili *et al*., 2001; Garcia *et al*., 2009). Phages have specific bacterial hosts and can infect bacteria without harming the plant or animal host of the bacterium (Fischetti, 2005). Unlike broad-spectrum antibiotics, phages also have no detrimental effect on mammalian cells (Jassim and Limoges, 2014). In developing a phage therapy, target phages are isolated from the natural environment of the bacterium (Chanishvili *et al*., 2001; Kelly *et al*., 2011). Then the screening process aims to identify a consistently lytic phage, robust enough for commercial use. Phage therapy was used in human medicine from the early 1900s but was abandoned in favour of the broader spectrum antibiotics in the 1940s (Jassim and Limoges, 2014). However, in Russia, Georgia and Poland phage research for use in human medicine has continued to the present day (Jassim and Limoges, 2014). The development of antibiotic resistance has increased the need to find alternatives to antibiotics. One of the widely researched phage therapies is Phagoburn, which was funded by the European Union, where phages are used to treat opportunistic bacteria infecting burn wounds (Soothill, 1994; Jassim and Limoges,

2014). Bacterial resistance is prevented by applications of cocktails of multiple phages, which reduces the probability of bacterial resistance developing to the phages (Jassim and Limoges, 2014). Phage therapy can be administered in accordance with the infection, either topically, intravenous, or orally (Chanishvili *et al*., 2001). Once a phage therapy is administered, the phages replicate exponentially in hours inside target bacteria, unlike the antibiotic counterpart which requires multiple applications (Chanishvili *et al*., 2001). Phages do not produce toxic residues/metabolites after treatment, and once the target host is destroyed, the phages will be dispersed harmlessly (Kelly *et al*., 2011). Bacterial resistance is overcome by phages through synergistic mutations with the host bacterium in the natural environment (Kelly *et al*., 2011). Phages show a very low frequency of triggering an allergic reaction in humans and can be administered to humans who are allergic to antibiotics (Chanishvili *et al*., 2001). The ability of phages to eliminate an antibiotic-resistant infection was demonstrated by Smith and Huggins in 1982. A single administration of a phage to treat lethal intramuscularly induced infection of mice with *Escherichia coli* demonstrated greater therapeutic potential to cure the bacterial infection than multiple intramuscular doses of tetracycline, ampicillin, chloramphenicol, or trimethoprim plus sulphafurazole (Smith and Huggins, 1982).

1.10.1 The Economic Importance of Phage's

The latest market report published by Credence Research estimated the bacteriophage industry of clinical application, food & beverages, phage display, phage therapy, environmental application and veterinary (Credence Research, 2018). To be valued at US$ 567.9 Million in 2017 and forecasted to grow by compound annual growth rate of 3.9% from 2018 to 2026 (Credence Research, 2018). The Credence finical report listed AmpliPhi Biosciences Coperation, EnBiotiz, Phage Biotech Ltd, Fixed-Phage Limited, InnoPhage, Ltd., Pherecydes Pharma, TechnoPhage, Lysando GmbH and Versatile as the key market players (Credence Research, 2018). The largest sector regarding revenue is reported to be food and beverage industry, with application of food biocontrol of pathogenic bacteria in ready to eat meals, fruits and dairy products (Credence Research, 2018). The geographic distribution of these

companies is spread Europe, North America, Asia pacific, Latin America, Middle East and Africa (Credence Research, 2018).

1.10.2 Phage Mode of Action

Phages are further divided by their life cycle into either lytic or temperate (Basdew and Laing, 2011). In the lytic cycle, virions inject their genome into bacterial cells, the host's machinery is used to replicate, and the host cells are lysed, releasing the next phage generation (Parisian *et al*., 2008; Madigan *et al.*, 2012).

Temperate phages differ because the viral genome is either assimilated into the host genome or remains as a plasmid in the host (Parisian *et al*., 2008; Madigan *et al.,* 2012). Instead of replicating, it stays dormant (prophage). It may remain in this prophage state indefinitely, failing to replicate inside the host cell (Parisian *et al*., 2008; Basdew and Laing, 2011). These cells are referred to as lysogenic, having the potential of producing lysis (Madigan *et al*., 2012). Eventually, the prophage host cell will enter a lytic cycle of the viral genome replicating and releasing phages (Parisian *et al*., 2008).

1.10.2.1 Absorption

Phage tails have specialized attachment structures that bind to specific surface molecules of a bacterial host (Basdew and Laing, 2011). The receptors of Gram-negative bacteria vary from oligosaccharides, proteins, teichoic acid and lipopolysaccharides (Parisian *et al*., 2008; Basdew and Laing, 2011). The peptidoglycan layer encapsulating Gram-positive bacteria offers multiple binding sites (Parisian *et al*., 2008). Some phages require two different surface molecules to be present before binding, due to phage tail attachment receptors (Parisian *et al*., 2008). Bacterial cells may become resistant to strains of phages by changing the structure of the surface molecule that a particular phage bind to (Vinodkumar *et al*., 2005). However, phages can compensate by a range of mutations, which adapt the tail fibres to recognize the altered surface molecules of the host bacterial cells (Vinodkumar *et al*., 2005).

1.10.2.2 Penetration

After attachment of phage tail fibres to bacterial surface molecules, the viral genome is inserted into the host cell (Parisian *et al*., 2008). The mechanism of viral genome transfer varies among phage species (Elbreki *et al*., 2014). Enzymatic reactions allow the viral genome to pass through the peptidoglycan and membrane layers of the host cell (Parisian *et al*., 2008; Elbreki *et al*., 2014). The viral genome is then incorporated into the host cell genome. This process is largely unknown for most phages (Parisian *et al*., 2008). However, once the viral genome has been injected into the bacterial cell, it is subjected to exonuclease and restriction enzymes (Parisian *et al*., 2008; Elbreki *et al*., 2014). Phages have overcome recognition by these enzymes by altering their genetic sequences (Parisian *et al*., 2008; Elbreki *et al*., 2014). Sticky ends or terminal redundancies allow the sequence to either circularize or remain linear, thereby avoiding enzymatic digestion (Elbreki *et al*., 2014).

1.10.2.3 Replication

Phage promoters lead RNA polymerase of the host to recognize phage genes and the transcription products restructure the host for the replication of phages (Parisian *et al*., 2008; Elbreki *et al*., 2014). In the process, the phage transcripts deactivate the host's cellular functions: macro-molecular biosynthesis, enzymatic action (proteases, restriction enzymes) and protein functions (Madigan *et al*., 2012). Phages either encode for their own RNA polymerase or for DNA-binding proteins that reprogram the host's RNA polymerase (Madigan *et al*., 2012). The next set of phage genes transcribed are involved in the synthesis of phage components (Madigan *et al*., 2012). DNA is then packed into the phage structures (icosahedral protein shells), aided by scaffolding proteins, after which the phage tails are attached and assembled separately (Madigan *et al*., 2012). Once the phages are assembled, their final step is lysis of the host cell (Madigan *et al*., 2012). Tailed phages use the enzymes lysin and holin to exit the host cell (Parisian *et al*., 2008; Elbreki *et al*., 2014). Lysin is an enzyme that breaks down the bonds in the bacterial peptidoglycan layer (the cell wall) and holin is a protein that creates pores in the inner membrane of the bacterial cell, allowing lysin enzymes passage through the inner membrane to the cell wall (Parisian *et al*., 2008; Elbreki *et al*., 2014).

1.10.3 Phage Characterization

Phages are prokaryote viruses of one order and are currently subdivided into 17 families and three floating groups, as listed in Table 1.1 (Ackermann and Prangishvili, 2012). Phage particles are constituted of a DNA or RNA genome, encapsulated by a protein or lipoprotein coat known as the nucleocapsid (Ackermann *et al*., 2009). The genome of phages ranges from thousands of base pairs to 480,000 base pairs (Ackermann and Prangishvili, 2012). The largest genome of a phage that has been sequenced is Phage G with a genome size of 700kb (Ackermann and Prangishvili, 2012). Viruses are also differentiated by shape (rods, spherical, lemon-shaped or pleiomorphic), and their capsid, genome and lytic abilities (Ackermann *et al*., 2009; Ackermann and Prangishvili, 2012). Over 95% of phages belong to the order Caudovirales, which contains three main families, *Myoviridae*, *Siphoviridae*, and *Podoviridae*, which are further differentiated by their specific tail morphology (Figure 1.2) (Ackermann *et al*., 2009). The other 14 families contain filamentous, cubic and pleomorphic phages with single/double-stranded DNA or RNA (Ackermann *et al*., 2009). The International Committee on Taxonomy of Viruses uses every set of defining characteristic to classify phages (Ackermann *et al*., 2009; Madigan *et al.*, 2012). The taxonomic names are derived from Latin or Greek dialect (Madigan *et al.*, 2012). Order, family and genera end in -*virales*, -*viridae* and -*virus*, respectively (Madigan *et al.*, 2012).

Table 1.1 Characteristics of the 17 families and 3 floating groups of phages (Ackermann, 2007)

Shape	Nucleic acid	Virus group	Particulars	Example
Tailed	DNA, 2, L	*Myoviridae*	tail contractile	T4
		Siphoviridae	tail long, noncontractile	λ
		Podoviridae	tail short	T7

Polyhedral	DNA, 1, C	*Microviridae*	conspicuous capsomers	ϕX174
	2, C, S	*Corticoviridae*	complex capsids, lipids	PM2
	2, L	*Tectiviridae*	inner lipid vesicle, pseudotail	PRD1
	2, L	SHI, group*		SH1
	2, C	STV1 group*	inner lipid vesicle	STIV
	RNA, 1, L	*Leviviridae*	turret-shaped protrusion	MS2
	2, L, seg	*Cystoviridae*	poliovirus-like envelope, lipids	Φ6
Filamentous	DNA, 1, C	*Inoviridae*	(1) long filaments	(1)fd
	2, L	*Lipothrixviridae*	(2) short rods	(2)MVL1
	2, L	*Rudiviridae*	envelope, lipids	TTV1
			TMV-like	SIRV-1
Pleomorphic	DNA, 2, C, S	*Plasmaviridae*	envelope, lipids, no capsid	L2
	2, C, S	*Fuselloviridae*		SSV1
	2, L, S	*Salterprovirus*	same, lemon-shaped	His1
	2, C, S	*Guttavirudae*	same, lemon-shaped	SNDV
	2, L	*Ampullaviridae**	droplet-shaped	ABV
	2, C	*Bicaudaviridae**	bottle-shaped	ATV
	2, L	*Globuloviridae**	two-tailed, growth cycle paramyxovirus-like	PSV

C: Circular; *L*: linear; *S*: superhelical; *seg*: segmented; *1*: single-stranded; *2*: double-stranded, ∗Awaiting classification.

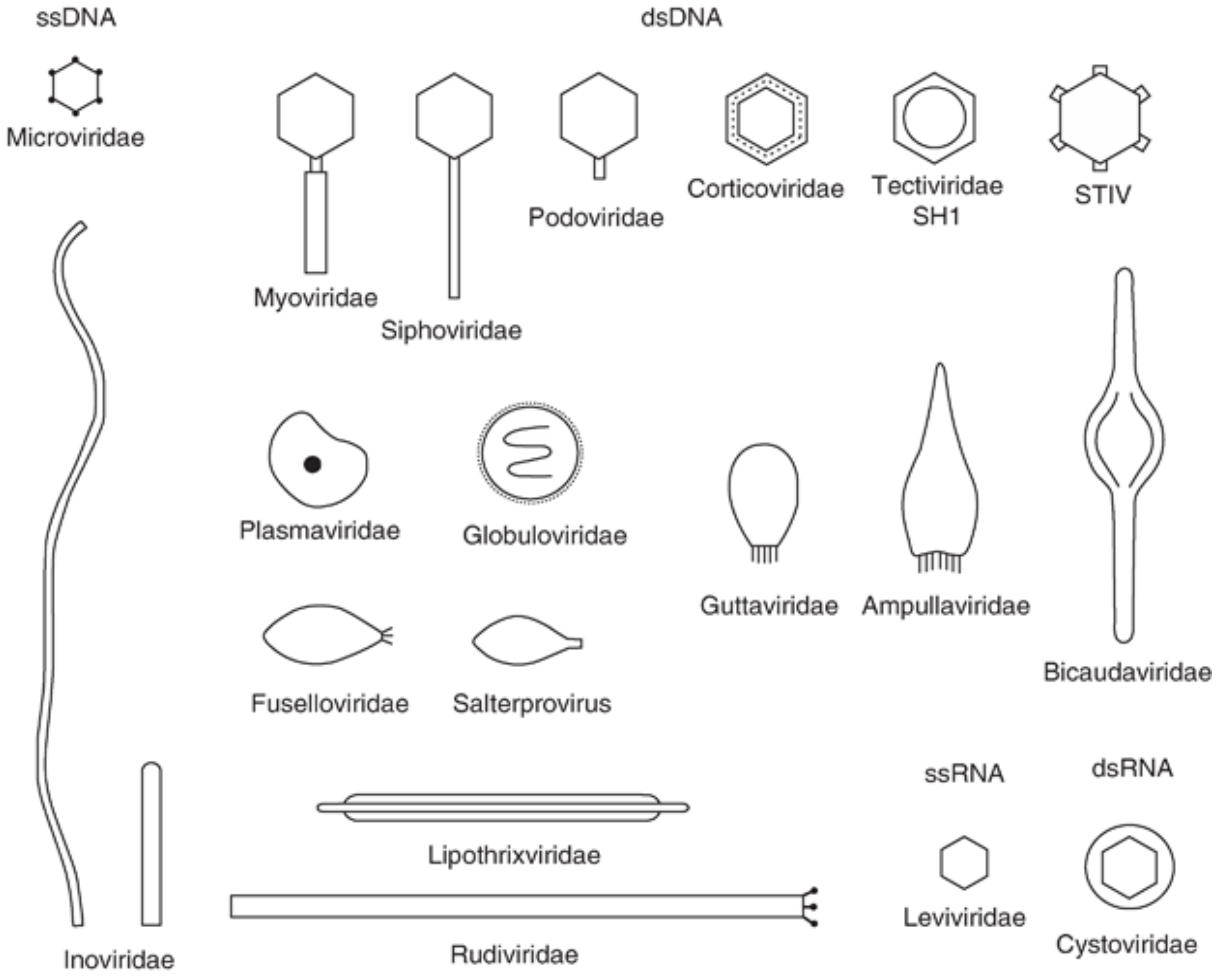

Figure 1.2 Phage shape of each family and floating groups (Ackermann, 2007).

1.11 Diagnosis Methods

1.11.1 Electron Microscopy Identification

Electron microscopy (EM) has facilitated research into virus structure, their entry into cells and their assimilation during replication (Ackermann and Prangishvili, 2012). This technique is used globally for the diagnosis and pathogenesis studies of viruses (Ackermann and Prangishvili, 2012).

For diagnostic purposes, this technique is less reliable when the virus particles are present in low concentrations in the host. This limiting factor is circumvented by artificial bulking of the virus before viewing samples (Ackermann and Prangishvili, 2012). In cases of viruses being difficult to locate in samples, an immunosorbent electron microscopy technique is used which employs serology to aggregate virus particles (Madigan *et al.* 2012). Grids are coated with the corresponding antibodies of the virus that increase the sensitivity and selectivity of virus particles present (Madigan *et al.* 2012).

The added benefit of EM is seen when identifying unknown virus particles because no organism specific reagents are required, unlike other serological tests that require specific probes to identify the presence of a virus (Madigan *et al.* 2012). This is helpful because serological identification is not available for all phages. The detection and differentiation of different particles have been successfully accomplished for different phage families (Ackermann and Prangishvili, 2012). Viral families such as *Myovridae, Siphoviridae* and *Podoviride* in Figure 1.2 have easily distinguishable virus particles due to their distinct particle morphology (Ackermann and Prangishvili, 2012).

1.11.2 Genomic Identification of Pathogens

Molecular techniques for pathogen identification started to be developed in the late 1980s with the development of DNA amplification techniques used to identify disease-causing pathogens (Clarridge, 2004). The amplification of conserved regions of DNA allowed for the identification and differentiation of pathogens down to the species level (Janda and Abbott, 2007). Conserved regions of DNA are part of an organism's genetic code that is generally similar or identical sequences in nucleic acids (DNA and RNA) across species (Janda and Abbott, 2007). The development of the 16s rRNA primers has allowed for a useful means of DNA amplification identification, due to the highly conserved nature of this gene (Woo *et al*., 2008). The 16s rRNA gene is used because of its existence in most, but not all, bacterial species (Woo *et al*., 2008). The gene function has not changed, hence, random variations in the sequence can be used as evolutionary markers (Woo *et al*., 2008).

Growing amount of DNA sequencing data is being shared globally due to advancements in genomic technology. Highly conserved regions within given taxons have been coded, allowing for more specific primer selections to differentiate between isolates within the species level (Janda and Abbott, 2007; Woo *et al*., 2008). Viruses are genetically different from bacterium because they do not appear to have universal conserved regions and therefore “universal” primers cannot be developed for viral identification (Clarridge, 2004; Janda and Abbott, 2007). Specific primers are required for different classes of viruses because their conserved regions vary (Clarridge, 2004; Janda and Abbott, 2007).

Today DNA amplification (polymerase chain reaction) is used in conjunction with microbial culturing, microscopy and serological tests using antigen detection techniques. This is due to the lack of broad-spectrum primers used on pathogens outside of the bacterial order. An example of this is viruses with little to no conserved genetic material due to recombination and mutation of genomic material, which is increasing evident with RNA viruses (Clarridge, 2004).

Whole genomic sequences not only allow for the identification of a virus but also reveal the mutations and evolutionary changes of a virus (O'Flaherty *et al*., 2018). Due to the relatively small genomes of phages (thousands of base pairs to 480,000 base pairs) (Ackermann and Prangishvili, 2012), whole genome sequences of phages will be relatively easy to work with within statistical models (O'Flaherty *et al*., 2018). Polymerase chain reactions (PCR) has been the most commonly used virus detection method, as opposed to the whole genome sequencing (Next generation sequencing (NGS) (O'Flaherty *et al*., 2018), by amplifying a segment of the virus genome that is common to strains of a genus and comparing it to known DNA sequences from the National Center for Biotechnology Information (NCBI). An open source statistical package called the Basic Local Alignment Search Tool (BLAST) can then be used to identify sequence similarities with reference sequences on the NCBI database, identifying the virus (O'Flaherty *et al*., 2018).

1.12 Conclusion

The inevitable evolution of antibiotic-resistant pathogens has led to an increasingly search for viable alternatives to antibiotic therapy for the treatment of bovine mastitis.

Phages may have a role to play as a supplement of antibiotic therapy to manage bovine mastitis. Phage therapy was being developed in 1900 but was largely abandoned in favour of antibiotics in western countries when antibiotics became widely available in the 1940's. Currently, publications documenting the versatility and safety of phage therapy have been increasing in frequency every year. This is evident with the International Committee on Taxonomy of Viruses documenting the discovery of novel phage families of approximately 100 novel phages a year (Ackermann, 2007). Phages may not replace antibiotics immediately but may work as adjunctive therapy with antibiotics to increase the efficacy of treatment of mastitis. It also shows promise as a standalone treatment for both preventative and curative treatments of bovine mastitis.

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CHAPTER 2

Screening *Streptococcus uberis* strains isolated from dairy farms in KwaZulu-Natal, RSA for antibiotic resistance using the Kirby Bauer disc diffusion method

Antibiotic-resistant bacteria are recognized as a major public threat due to the recorded 2 million people infected yearly in the USA, with a minimum of 23,000 fatalities. In developing countries livestock production accounts for the bulk of antibiotic use, claiming 50-80% of consumption. Tracking the incidence of antibiotic resistance in mastitis-causing bacteria would be useful for the South African dairy industry. The aim of this chapter was to identify resistant *S. uberis* strains to access the phage therapy efficacy in chapter 3. Eleven strains of bacteria that were potentially identified as *S. uberis*, subsequently they were screened using 16s ribosomal RNA sequencing. Six strains were identified as *S. uberis*. These strains were screened against 8 antibiotics commonly used in the dairy industry and vancomycin. Using the Kirby Bauer method and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breaking points zone diameters, version 10.0, 2020. Of the following antibiotic families: β-lactam (ampicillin, penicillin G, cefalexin, oxacillin and amoxicillin), macrolides (erythromycin), tetracyclines (tetracycline) and glycopeptide (vancomycin). Strain 21A was resistant to 7 of the test antibiotics. All the strains were susceptible to vancomycin, which is not available for intra-mammary infections. Resistance to the 7 other antibiotics varied amongst the strains. The study was based on mastitis strains isolated from KZN, submitted by farmers. It represented only those who could afford veterinary diagnostics from Allerton Laboratory. This was not a survey study but an identification of resistant strains to challenge the phage therapy efficacy.

2.1 Introduction

The emergence of antimicrobial resistance has become a prominent feature on the international agenda of human and veterinary health (WHO, 2014). Antibiotics are produced by microorganisms as secondary metabolites to alleviate the competition for a food source in their natural environment (Marinho *et al*., 2016). Currently, antibiotics are found in the environment at abnormal levels relative to the sub-inhibitory quantities naturally present (Goh *et al*., 2002). In an ecosystem, apart from bacterial inhibition, antibiotic molecules trigger gene transfer, intra-chromosomal recombination, stress responses, biofilm formation and phage introduction, thus acting as signal molecules (Goh *et al*., 2002; Yim *et al*., 2007; Baquero *et al*., 2013).

In developing countries, livestock production accounts for most of the antibiotic use, with 50-80% of antibiotic consumption (Cully, 2014). Subsequently, the antibiotics enter the environment via effluent discharge, manure and sewage run-offs (Marinho *et al*., 2016). Similarly, the use of antibiotics on humans results in a release of antibiotics into the environment, wastewater systems, and then into large aquatic and marine systems (Marinho *et al*., 2016).

Resistance has developed because antibiotics are not fully metabolized in food production, and veterinary and human use, and are subsequently released into the environment, where selection for resistance takes place (Sarmah *et al*., 2006). Studies have documented bacterial cells possessing resistance to all classes of antibiotics prescribed, pre-empting the development of conservative antibiotic therapies (Cully, 2014; Marinho *et al*., 2016). The WHO is leading the appeal to governments and private institutions to acknowledge the antibiotic resistance epidemic resulting from overuse, and unregulated use worldwide (WHO, 2014). However, the emergence of antibiotic resistance is a natural consequence of evolution and is a function of the selection pressure imposed on bacteria (Marinho *et al*., 2016).

Micro-organisms are capable of horizontal gene transfer of resistance to phylogenetically distant species (Gillings and Stokes, 2012). They are also capable of transmitting resistance between different hosts in a multitude of ecosystems (Couce and Blázquez, 2009). The long-term consequences of releasing these antimicrobial resistant bacteria into the environment cannot be predicted without detection

programmes administered by governmental structures (Marinho *et al*., 2016). The aim of this chapter was to identify resistant *S*. *uberis* strains present in KwaZulu-Natal (KZN) to access the phage therapy efficacy in chapter 3.

2.2 Materials and methods

2.21 *Streptococcus uberis* identification and isolation

From June 2017 to February 2018, *S*. *uberis* strains isolated by Allerton Provincial Veterinary Laboratory from unpasteurized milk samples. On request of dairy farmers in the KZN region for total bacterial counts and bacterial identification analysis. Strains were subcultured onto blood agar and delivered to the University of KwaZulu-Natal laboratory. Allerton's laboratory identified *S. uberis* using the following tests: gram reaction, haemolysis patterns on blood agar, catalase reactions using hydrogen peroxide (5%), CAMP test (Christie-Atkins-Munch-Peterson) and an esculin hydrolysis as per their inhouse standard operating procedures.

The identity of the strains were then confirmed by sequencing of the 16s ribosomal RNA by Inqaba Biotechnical Industries (Pty) Ltd.

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers 16S-27F and 16S-1492R. The PCR products were run on a gel (**Appendix A**) and gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was used to analyse the generated sequences by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

2.22 Antibiotic resistance assay

The Kirby Bauer diffusion method described by Bauer *et al*. (1966) was used to screen *S*. *uberis* strains for resistance against four families of antibiotics commonly used to treat bovine mastitis in the dairy industry. The antibiotic families used were β-lactams (ampicillin, penicillin G, cefalexin, oxacillin and amoxicillin), macrolides (erythromycin), tetracyclines (tetracycline), and glycopeptides (vancomycin). All assays were carried out in triplicate (**Appendix B**), on tryptone soy agar (TSA) (100 mm diameter, 4mm agar thickness; pH 7.2) infused with a previously prepared bacterial culture (*S. uberis* in broth, incubated for 36 hours at 37°C). Prior to use, the poured Petri dishes were dried in an incubator at 37°C for 60 minutes to evaporate excess moisture. Plates were incubated for 36 hours at 37°C. Inhibition zones were measured in millimetres using a digital calliper (GS5071522, Mastercraft). Zones of inhibition were averaged (**Appendix C**) and resistance determined according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breaking points and zone diameters, version 10.0, 2020 (EUCAST,2020). Testing was performed at the University of KwaZulu-Natal, Pietermaritzburg laboratory.

2.3 Results

2.3.1 *Streptococcus uberis* isolation

From a collection of 26 bacterial isolates (identified as *S. uberis*) provided by Allerton Provincial Veterinary Laboratory, 11 isolates chosen at random and were submitted to Inqaba Biotechnical Industries (Pty) Ltd for 16s ribosomal RNA sequencing and identification, which revealed that 6 of the 11 strains were *S. uberis*. The reminder sequences were identified as *Streptococcus dysgalactiae, Brevibacillus centrosporus, Lactococcus garvieae* and *Bhargavaea cecembensis*.

2.3.2 Antibiotic resistance assays

Table 2.1 Antibiotic resistance screening of six *S. uberis* strains: 4B, 78B, 21A, 17D, 15D, 31C.

Antibiotic	Disc Concentration	*Streptococcus uberis* strains					
		4B	78B	21A	17D	15D	31C
Amoxicillin	10µg	S	S	R	S	S	S
Ampicillin	10µg	S	S	R	R	S	S
Cefalexin	30µg	S	S	R	S	S	R
Erythromycin	15µg	S	R	R	S	S	S
Penicillin G	10 units	S	S	R	S	R	S
Oxacillin	1 µg	S	S	R	S	S	R
Tetracycline	30µg	S	S	R	R	S	S
Vancomycin	30µg	S	S	S	S	S	S

S=susceptible to antibiotic, R=resistant to antibiotic

Five of the six strains of *S. uberis* that were screened was resistant to one or two antibiotics tested, which are all commonly used in the dairy community to treat *S. uberis* bovine mastitis (Table 2.1) Strain 21A possessed resistance to all the test antibiotics except vancomycin (Table 2.1).

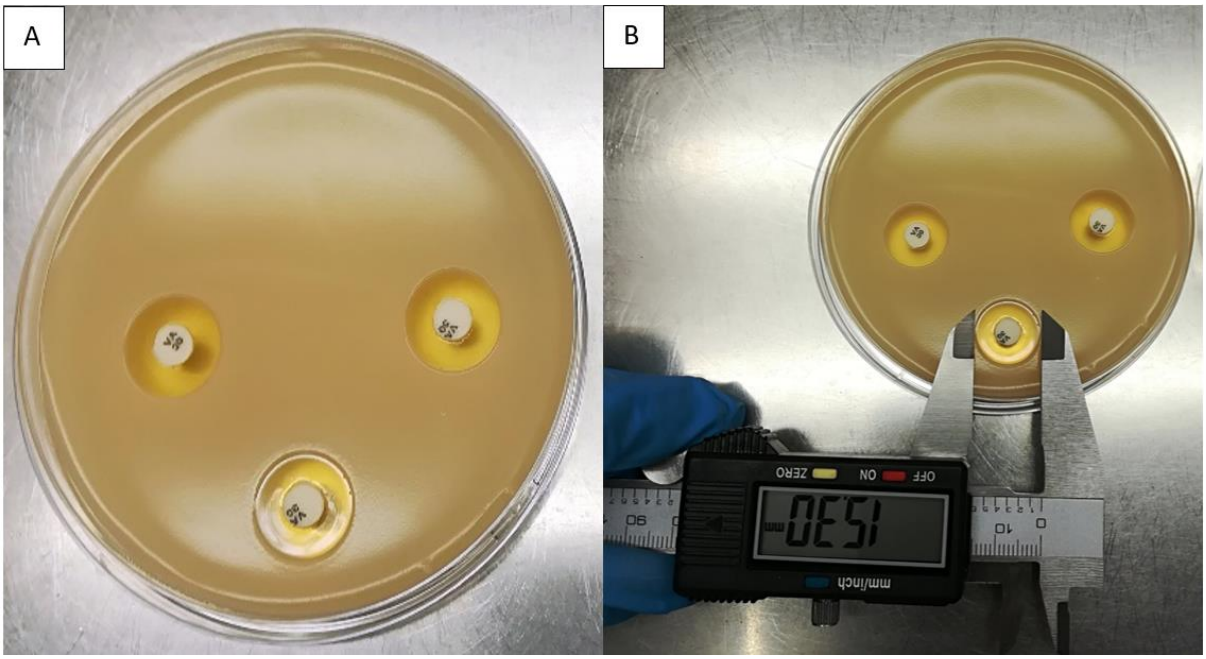

Figure 2.1 The disc diffusion method used to screen *S. uberis* strain 17D for resistance against vancomycin (30µg). A zone of 13mm or less would indicate that strain 17D was resistant to vancomycin according to EUCAST, version 10.0, 2020.

The measurements of the zone of inhibition were measured in millimetres to the second decimal point using a digital calliper, as illustrated in Figure 2.1.B. Zones of inhibition were rejected on the following basis: overlapping of zones, distortion of zones (irregular shape) and irregular growth of bacterium being screened. Figure 2.1.A illustrates a typical example of an acceptable zone of inhibition with a distinct circumference and even bacterial growth.

2.4 Discussion

Antibiotic resistance is a global problem including South Africa. It threatens health care of humans and animals in the control of bacterial infections. Strategies to manage it include tracking trends of shared resistance, identifying evolving resistance genomic resistomes, creating a freely accessible data base of resistomes and alerting of appropriate authorities (health care professionals) of epidemic levels of cases of novel resistance to previously effective antibiotics (Jones, 1999; Marinho *et al*., 2016).

Addressing the problem of zoonotic pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) in the global public health arena requires the integration of all stakeholders in an interdisciplinary collaboration (Chang *et al*., 2003). Resistant genetic material can be shared or acquired in an environment by bacteria, including via horizontal transfer between species and genera (Marinho *et al*., 2016). Resistant bacteria act as reservoirs of resistant genes for other bacteria, exacerbating a pre-existing problem (Grundmann *et al*., 2006). Hence, the classifying of the resistome is essential for the control of antibiotic-resistant causal agents of mastitis (Marinho *et al*., 2016). New resistance may emerge from gene deletions, gene gains, gene duplication, genomic sequence rearrangements, gene transfer and other chemical signals that alter sequences at the nucleotide level (Grundmann *et al*., 2006).

The emergence of multidrug-resistant pathogens is becoming more frequent across all industries that use antibiotics, which includes food and agricultural production, and human and animal medicine (Marinho *et al*., 2016). The WHO has identified multidrug resistance in *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp*.,* and *Enterococcus* spp. as a global concern (Grundmann *et al*., 2006; ECDC, 2013; WHO, 2014).

In the current study, resistance was found to 3 of the 4 families of antibiotics tested. Two strains were resistant to tetracycline, which may be related to similar resistance found in *E. coli* and enterococcal species predominantly found in the wild (Marinho *et al*., 2016). These results also correlate with a European Union report in 2013, based on data from 25 countries, that tetracyclines were the most purchased antibiotic class in the food production industry (European food safety authority and European centre for disease prevention and control, 2016). The current study also found ampicillin resistance in two of the six strains, unlike other studies that have found widespread resistance to this broad-spectrum antibiotic (Sayah *et al*., 2005). Due to the low number of isolates screened, a generalized deduction cannot be made for the region.

Vancomycin is an antibiotic used as a last resort to treat problematic bacterium such as methicillin-resistant *S. aureus*, yet its widespread use has led to the emergence of vancomycin resistance in *S. aureus*, with the gene responsible being *vanA* (Chang *et al*., 2003). The elimination of resistance genes from the environment is not possible. Theoretically, it would be desirable to prevent the creation of new resistance genes in

target bacteria and to limit the spread of current resistant genes into production environments, which would require surveillance/screening for resistance, reduced use of antibiotics that induce bacterial mutations, and the reduction of antibiotic-rich environments that currently allow for the horizontal transfer and recombination of genes, and the suppression of phenotypic resistance traits (Wright, 2009). This combination of requirements is complex and unlikely to occur in practice, so the development of antibiotic resistance is inevitable if correct udder management practises are not followed.

The current strategy to deal with antibiotic resistance is through the discovery of novel antimicrobial treatments. However, this has been unsuccessful due to the speed of resistance development, and the slow pace of discovery and registration of new antibacterial compounds, and the huge costs of novel drug registrations (Couce and Blázquez, 2009: Wright, 2009). Stopping the use of certain antibiotics for a period appeared to be a solution, but this was disproved in Sweden where *E. coli* resistance to trimethoprim was reported, followed by removal of the compound for two years (Brolund et al., 2010). However, this did not reduce the frequency of occurrence of the resistant gene *dfr*, and dissemination of the gene still occurred with co-selection occurring with other genes. Thus, antibiotic resistance will keep evolving to attain resistance to more and more antibiotics (Brolund *et al*., 2010).

Going forward, future studies could include sequencing the predominant strains of *S. uberis* determining the actual genes responsible for the antibiotic resistance. Bryan *et al*. (2014) showed that there are a multitude of genes that can be responsible for resistance to tetracycline, with three documented mechanisms: enzymatic inactivation of the antibiotic, efflux pump and ribosomal protection. Identifying the resistant genes present and the resistance mechanism could allow for strategic countermeasures to avoid multidrug resistance. The use of antibiotics in agriculture may have unprecedented consequences, and further studies are required to identify resistance patterns and genes present in the microbial community to moderate the conditions that lead to resistance. Identifying the resistant genes present and the resistance mechanism would allow for an understanding of the process and scale of multidrug resistance. The use of antibiotics in agriculture may have unprecedented consequences, and further studies are required to identify resistance patterns and

genes present in the microbial community to model the future development of resistance and to develop contingency plans for a future where there are few, if any, effective antibiotics.

The shortcomings in this report, the study was based on mastitis cases from KZN reported by farmers. It represented only those who could afford veterinary diagnostics from Allerton Laboratory. With 6 strains being evaluated and these findings are not generalizable to the entire region or country. This was not a survey study but an identification of resistant strains to challenge the phage therapy efficacy. Animals that were on medication could have affected the low recovery of bacterial isolates.

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CHAPTER 3

Biological and pathogenicity assays of phages specific for *Streptococcus uberis* strains, associated with bovine mastitis

Mastitis is the most economically important disease to dairy herds worldwide, with losses arising from decreased milk production, and increased veterinary bills and culling. Studies in sub-Saharan Africa revealed that at least 50% of dairy cows were infected. The current study isolated six *Streptococcus uberis* phages and then and characterized for the following parameters: phage titre, host range, single step growth phase and lethal dose activity. Approximately 2,000 mastitic milk samples were screened for phages, from which 95 phages were isolated. This number was reduced by screening down to 5 phages, Phages Numbers CP1, 2, 76, 79 and 80. Phages CP1, CP2, CP76, CP79 and CP80 produced titres of $59x10^2$, $56x10^5$, $47x10^6$, $50x10^4$ and $35x10^4$ $pfu.ml^{-1}$, respectively. In the strain susceptibility assay there was considerable specificity between the phages and strains of *S. uberis*. One strain of *S. uberis* was susceptible to all five phages, whereas Strains 17D and 78B were not susceptible to any of the five phages. Phage CP2 was virulent to four of the six *S. uberis* strains. A single step growth assay illustrated that the cyclic replication of the isolated phages took between 50 and 60 minutes. In the lethal dose assay, Phages CP1 and CP2 were each able to reduce *S. uberis* counts by 86% and 83%, respectively. Of the 5 phages screened, Phages CP1 and CP2 showed significant potential as a stand-alone treatment. However, Phages CP76, CP79 and CP80 would offer sufficient control in a phage cocktail.

3.1 Introduction

Bovine mastitis is an inflammation of the parenchyma of mammary glands of dairy cows (Jones and Bailey, 2009). The symptoms comprise of physical and chemical changes of the glandular tissue and of milk, adversely affecting the cow's health and quality of milk (Blowey and Edmondson, 2010). The bacteria causing bovine mastitis are usually located on the teats of the cow, which then serve as a reservoir of infection

from which to spread from one cow to the next during milking, or they are introduced to the teats of the cow from the environment (Blowey and Edmondson, 2010).

S. uberis is extremely versatile and able to survive in various areas of the cows, mouth, vulva, groin and axilla (Almeida *et al*., 2006; Blowey and Edmondson, 2010). The bacterium has been identified as an environmental pathogen and is found in the bedding (straw and sand), pastures and faeces (Almeida *et al*., 2006; Blowey and Edmondson, 2010). *S. uberis* is the most common pathogen causing environmental mastitis in most countries, due to its poor opsonization in the presence of casein protein and its ability to exist inside cells, which means that it is protected from antibiotics (Hillerton and Kliem, 2002).

Phage therapy researched as a biological control, targets a specific strain of bacterium and is harmless to the host and beneficial bacterium (Basdew and Laing, 2011). Phages are naturally occurring viruses that are a part of the microbial ecosystem, with the natural environment containing billions of phages (Chanishvili *et al*., 2001; Garcia *et al*., 2009). Numerous phage applications on food items are currently being developed for commercial use (Sofos, 2008). A *Salmonella* phage treatment has been commercialized to control growth of *Salmonella* on cooked and raw beef (Sofos, 2008). Such applications of phages as a biocontrol strategy is set to become an increasingly common practice in the global food industry.

The aim of this chapter was to isolate phages of *S. uberis* and then to characterize them for the following parameters: phage titre, host range, single step growth phase and lethal dose activity. This *in vitro* study was an essential step towards phage therapy against South African strains of streptococcal etiological agents of bovine mastitis.

3.2 Materials and methods

3.2.1 *Streptococcus uberis* identification and isolation

From June 2017 to February 2018, *S. uberis* strains isolated by Allerton Provincial Veterinary Laboratory from unpasteurized milk samples. On request of dairy farmers in the KZN region for total bacterial counts and bacterial identification analysis. Strains

were subcultured onto blood agar and delivered to the University of KwaZulu-Natal laboratory. Allerton's laboratory identified *S. uberis* using the following tests: gram reaction, haemolysis patterns on blood agar, catalase reactions using hydrogen peroxide (5%), CAMP test (Christie-Atkins-Munch-Peterson) and an esculin hydrolysis as per their inhouse standard operating procedures.

The identity of the strains were then confirmed by sequencing of the 16s ribosomal RNA by Inqaba Biotechnical Industries (Pty) Ltd (http://www.inqababiotec.co.za).

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers 16S-27F and 16S-1492R. The PCR products were run on a gel and gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was used to analyse the generated sequences by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

3.2.2 Phage isolation, propagation and purification

During the period of June 2017 to February 2018 approximately 2000 milk samples were screen for phages. Weekly samples of milk were collected from Allerton laboratories which have been submitted by dairy farmers in the KwaZulu-Natal region for testing. Milk samples were collected by the farmers using aseptic techniques and transported to Allerton via cold storage, samples were screened for the requested tests and only samples identified as mastitic by Allerton were submitted to UKZN laboratories for the following study.

Milk samples were tested using the double-layer spot-plate technique for each *S. uberis* strains isolated (Sambrook *et al.,* 1989). Overnight *S. uberis* cultures were

incorporated into 0.7% top agar of a double-layer plate and supplemented with 1M $CaCl_2$. Raw milk was then spotted onto the surface of solidified top agar at 10µl per spot. Plates were allowed to dry for 2hr and then incubated at 37ºC x 36hr. Zones of clearing (plaques) were indicative of phage lytic activity. Plaques were removed and soaked in a 40% glycerol solution at 20ºC x 12hr with gentle agitation at 150rpm. The suspension was then centrifuged at 10,000g x 10min at 4ºC, and the supernatant was filtered using a 0.45µm syringe filter and stored as phage stock at 4ºC.

3.2.3 Phage titre

Phages were titred for each *S. uberis* strain isolated, using the double-layer spot-plate technique as described by Deininger, (Deininger, 1990). Overnight *S. uberis* cultures were incorporated into 0.7% top agar supplemented with 1M $CaCl_2$. For each phage, dilution series (10^0 to 10^{-10}) were prepared and incorporated into the top agar. Plates were allowed to dry for 2hr and then incubated at 37ºC x 36hr. Zones of clearing (plaques) were indicative of phage lytic activity. The number of plaque forming units per millilitre ($pfu.ml^{-1}$) was calculated as described by Deininger, (Deininger, 1990) where $pfu.ml^{-1}$ = number of plaques x reciprocal of dilution x dilution factor.

3.2.4 Phage host range analysis

Host range analysis was carried out using the spot-test method on double-layer plate. An overnight culture of each of the 6 *S. uberis* strains was incorporated into pre-warmed top agar (0.7%), supplemented with $CaCl_2$. Top agar was immediately poured into previously prepared bottom layer agar. Undiluted phage stock was screened against the *S. uberis* species by spotting 10µl droplets of each phage onto the surface of the *S. uberis* inoculated double-layer plate. All plating was carried out in triplicate. The plates were left to dry for 2hr followed by incubation for 36hr at 37ºC. Any plates exhibiting zones of the clearing were regarded as a positive result.

3.2.5 Single step growth curve

The protocol was carried out according to Harley and Prescott (1993). Phage stock solution was made up to a 10^{-1} dilution in 9.9ml of tryptone soy broth (TSB). Then 100 µl of *S. uberis* strain 21A was added to the TSB phage mixture and incubated at 37ºC with gentle agitation (150rpm). The control treatment consisted of 9.9ml TSB + 100 µl of *S. uberis*. Following 25 minutes of incubation, and at 5 minute intervals until 60 minutes (25min, 30min, 35min, 40min, 45min, 50min, 55min and 60min), 1ml of the TSB mixture was aliquoted into pre-warmed top agar (0.7%) of a double-layer plate supplemented with $CaCl_2$ and inoculated with an overnight culture of *S. uberis* strain, 21A. Top agar was immediately poured into the previously prepared bottom layer agar. Plates were allowed to dry for 2hr and then incubated at 37ºC x 36hr. Zones of clearing (plaques) were indicative of phage lytic activity. The number of plaque forming units per millilitre ($pfu.ml^{-1}$) was calculated as described by Deininger, (Deininger, 1990), $pfu.ml^{-1}$ = number of plaques x reciprocal of dilution x dilution factor.

3.2.6 Lethal dose assay

An overnight culture of *S. uberis* strain, 21A, was prepared in TSB and 50ml each was aliquoted to six sterile conical flasks. Bacterial cell counts per millilitre were determined after overnight incubation. Thereafter, each flask was inoculated with 5ml of the six phage stock solutions (Phages CP1, CP2, CP76, CP79 and CP80) and incubated for 24hrs at 37ºC. After incubation, live bacterial cell counts were determined using dilution plating.

3.3 Results

3.3.1 *Streptococcus uberis* and phage isolation

Eleven strains of *S. uberis* were isolated and identified. The identity of the 11 strains were then genetically confirmed by sequencing the 16s ribosomal RNA, which revealed that the six of 11 strains were *S. uberis* (4B, 78B, 21A, 17D, 15D and 31C).

Approximately 2,000 mastitic milk samples were screened for phages, from which 95 phages were isolated. From these, five test phages were selected for their activity.

3.3.2 Phage titre

Phages CP2 and CP76 produced higher plaque counts than the phages CP1, CP79 and CP80, suggesting that they are more virulent in nature. Plaque counts were attained at different dilution factors of the five phages screened, Phages CP1, CP2, CP76, CP79 and CP80 produced titres of $59x10^{2}$, $56x10^{5}$, $47x10^{6}$, $50x10^{4}$ and $35x10^{4}$ $pfu.ml^{-1}$, respectively. Phage CP1 produced the lowest phage titre and Phage CP76 produced the highest titre, demonstrating a difference in their replication and virulence. Acceptable plaque counts were between 20 and 90 plaques per plate.

3.3.3 Host range analysis

Zones of clearing (plaques) were indicative of phage lytic activity, whereas the absence of a plaque was indicative of no virulence by the phage to that strain of *S. uberis*. *S. uberis* 21A was susceptible to the five test phages (Table 3.1). In contrast, Strains 17D and 78B were not susceptible to the five phages. Phage CP2 possessed the largest host range, being infectious to four of the six *S. uberis* strains.

Table 3.1 Host range assay of five phages on *S. uberis* strains: 4B, 78B, 21A, 17D, 15D, 31C isolated from mastitic milk.

Phages	*Streptococcus uberis* strains					
	4B	**78B**	**21A**	**17D**	**15D**	**31C**
Phage CP1	No plaque	No plaque	Plaque	No plaque	Plaque	Plaque
Phage CP2	Plaque	No plaque	Plaque	No plaque	Plaque	Plaque
Phage CP76	Plaque	No plaque	Plaque	No plaque	No plaque	No plaque
Phage CP79	No plaque	No plaque	Plaque	No plaque	Plaque	No plaque
Phage CP80	Plaque	No plaque	Plaque	No plaque	Plaque	No plaque

3.3.4 Single step growth curve

Each phage possessed a varied rate of replication per millilitre (pfu.ml^{-1}) (Fig 3.1). Phages CP76 and CP2 developed the highest pfu.ml^{-1} at 60 minutes, whereas Phage CP80 developed the lowest pfu.ml^{-1} but reproduced the most rapidly, starting the exponential phase at 30 minutes. In contrast, Phage CP76 reproduced the slowest, only entering the exponential phase at 35 minutes, but produced the highest pfu.ml^{-1}. Each phage had a clear, single step growth cycle lasting between 50 and 60 minutes.

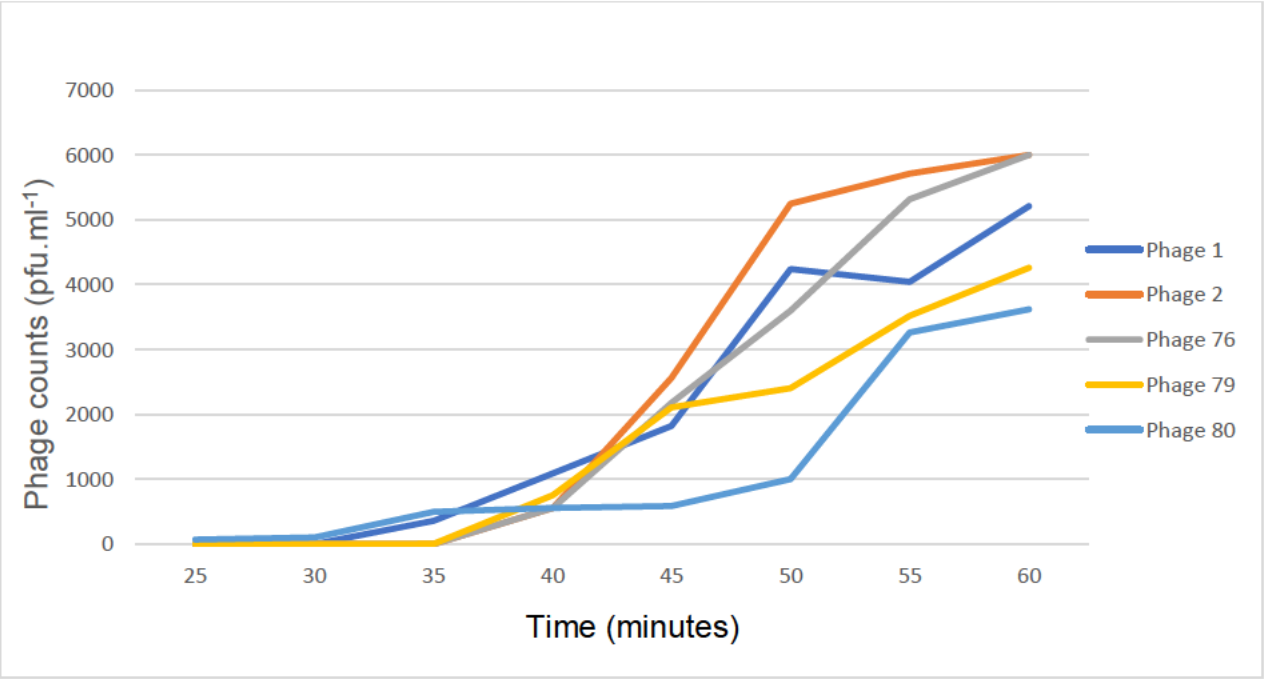

Figure 3.1 Infection curve for five *S. uberis* phages. The vertical bar represents phage count x time combination at 5-minute intervals.

3.3.5 Lethal dose

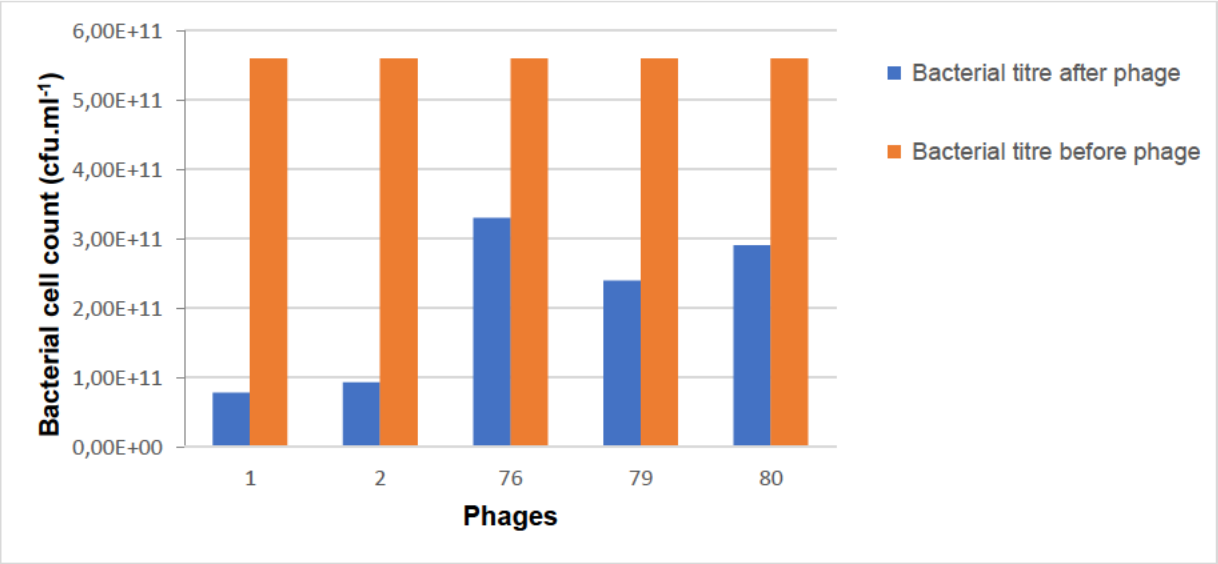

Figure 3.2 Lethal dose assay of *S. uberis* (Strain 21A) growth in TSB, with and without the addition of isolated *S. uberis* phages.

Bacterial titre before phage represents the initial *S. uberis* bacterial counts without phage addition and bacterial titre after phage refers to the addition of Phages (CP1, CP2, CP76, CP78 and CP80) and 24-hour incubation. Phages CP1, CP2, CP76 and CP80 reduced the initial bacterial counts by greater than half the original bacterial count (Figure 3.2).

Table 3.2 Reduction in live bacterial cell count after addition of phages to *S. uberis* Strain 21A, relative to the control illustrated in **Figure 3.2**.

Phage	**Percentage reduction in the growth of *S. uberis* (Strain 21A) cells**
Phage CP1	86 %
Phage CP2	83 %
Phage CP76	41 %
Phage CP79	57 %
Phage CP80	48 %

Phages 1 and 2 caused the highest reduction of live bacterial cells, 86 % and 83%, respectively, over a 24-hour period.

3.4 Discussion

Mastitis is a complex disease involving various factors and identifying the main pathogens and risk factors is fundamental to developing preventive and control measures (Harding, 1995; Van den Borne, 2010). *S. uberis* is capable of both clinical

and subclinical infections of the cows (Almeida *et al*., 2006). The cows are constantly exposed to *S. uberis* due to the ubiquity of the pathogen and once infected the pathogen may elicit a contagious infection (Almeida *et al*., 2006). *S. uberis* has been documented to show little responsiveness to antibiotic treatments (Burvenich *et al*., 2003; Blowey and Edmondson, 2010). Use of antibiotics in the treatment of mastitis increases the probability of creating antibiotic-resistant strains requiring alternative therapies (Mangili *et al*., 2005; Shi *et al*., 2010). One such measure is the use of biological control to treat mastitis, specifically the use of phage therapy to treat pathogenic bacterial infections (Garcia *et al*., 2009; Basdew and Laing, 2011).

The five phages in the current study were selected based on their consistent lytic ability after multiple sequential generations. These five phages were assessed for their ability to lyse mastitis-causing strains of *S. uberis* isolated from raw milk in the following assays: phage titre, host range assay, single strep growth phase and the death phase.

In the phage tire assay, a range of titres were produced by the five phages ranging from $59x10^2$ to $47x10^6$ for Phages CP2 and CP76, respectively.

The host range analysis revealed a differential interaction between the phages and the strains of *S. uberis*. Whereas Strain 21A was susceptible to all five phages, Strains 78B and 17D were not susceptible to any of the test phages. Phage CP2 was lytic on four of the six strains of *S. uberis*.

The single step growth assay tracked the replication cycle of the phages. At the start of the assay the $pfu.ml^{-1}$ was constant, but at 30 to 35 minutes, an exponential increase in $pfu.ml^{-1}$ occurred. Results suggested that after 50-60-minutes of incubation, phages lyse their hosts and are released (Gong *et al*., 1996).

In the lethal dose assay, Phages CP1 and CP2 were able to reduce *S. uberis* counts by 86% and 83%, respectively, over 24 hours, suggesting that a cocktail of phages may offer a more comprehensive therapeutic solution to an *S. uberis* bovine infection.

Of the 5 phages, Phages CP1 and CP2 showed significant potential as a stand-alone treatment. However, Phages CP76, CP79 and CP80 could be combined in a phage cocktail to increase their activity, and to broaden the host range of the therapy. Further characterization of the phages durability is required for the purposes of commercial

production. Sensitivity to variations in temperature and pH, and to chloroform and glycerol should be evaluated for each phage. Knowledge of the genomic identity of the phages would also be useful. Following the durability and genomic characterization, field trials would be required to evaluate the efficacy of each phage therapy as a treatment for strains of *S. uberis* causing bovine mastitis.

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CHAPTER 4

Morphological classification of phages specific to *Streptococcus uberis*, associated with bovine mastitis

Phages are viruses that exclusively infect bacterial cells. Phages are ubiquitous (found in all biospheres) and are the most abundant organisms on earth. Understanding the interaction between phages and their hosts is vital to their manipulation for therapeutic conditions. This study morphologically characterizes phages specific to *S. uberis*. Phages were isolated using the double-layer agar plate technique from 2,000 screened mastitic milk samples farms in KwaZulu-Natal, SA. Phage samples were propagated on *S. uberis* and examined on a transmission electron microscopy (JEOL 1400). Negative staining was used to view the virus particles. A virus particle image captured illustrated *Podovirdae* morphology, measured to have a 50-65nm diameter icosahedral head and a short tail ranging from 25-35nm in length. With a uniform structure observed, the phages isolated may belong to one family. The distinct structure of the phage tails was obscured in majority of the micrographs. However, this does not imply that no tail is present. It is probable that due to the complexity of the camera colour scaling and the low contrast that had to be applied, 2D images of the tail were not clear. Due to the poor quality of micrographs obtained attributed to the clumping of phages and cell exudate, genomic identification would be required to confirm the identity of these virus particles.

4.1 Introduction

The electron microscope has been used extensively as a diagnostic tool for identifying viral agents since the 19th century, with the first (confirmed) visualization of a virus performed by Ruska and colleagues Kausche and Pfankuch (Ackermann, 2007). Apart from the early visualization of significant viruses such as smallpox, chickenpox, poliovirus and gastrointestinal viruses, host and viral interactions were observed, leading to the discovery of treatments and prevention of these prior unseen disease

agents (Goldsmith and Miller, 2009). In 1976 during the haemorrhagic fever pandemic in then Zaire (DRC), the electron microscope was instrumental in identifying the Ebola virus (Johnson *et al*., 1977). Similarly, the Henipavirus was identified microscopically during an outbreak in both Asia and Australia (Chua *et al*., 2007).

Today, electron microscopy still offers an advantage over serological methods. Viral-specific reagents are not required for a negative stain, which is particularly applicable in the case of unknown or newly discovered viruses, where it would not be possible to obtain viral specific probes (Goldsmith and Miller, 2009). The list of viral infections presently identifiable solely by the electron microscope includes lymphocytic choriomeningitis virus, coronavirus and poxvirus (Bayer-Garner, 2005; Lin *et al*., 2004). A wide range of samples can be screened, from bodily fluids (urine, blood, spinal fluid, bronchiolar fluid, tears, aspirates) to organ tissue, and samples can be prepared within minutes via the negative staining procedure (Ackermann, 2007). Although assays such as ELISA and PCR are more sensitive and accurate, both require specific reagents and take hours to complete, in comparison to the rapid screening offered by electron microscopy. This allows electron microscopy to be a first line of diagnosis in time-sensitive circumstances such as bio-terrorism threats, terminal illnesses and diseases epidemics (Goldsmith and Miller, 2009). However, there are limitations to what the electron microscope can achieve. Viruses cannot be identified beyond the family level, and multiple viral infections are troublesome to diagnose (Vale *et al*., 2010). However, the results derived will allow for accurate molecular diagnostic assays to be chosen to identify the virus to a species level (Ackermann, 2007; Vale *et al*., 2010).

4.2 Materials and Methods

4.2.1 *Streptococcus uberis* identification and isolation

From June 2017 to February 2018, *S. uberis* strains isolated by Allerton Provincial Veterinary Laboratory from unpasteurized milk samples. On request of dairy farmers in the KZN region for total bacterial counts and bacterial identification analysis. Strains were subcultured onto blood agar and delivered to the University of KwaZulu-Natal laboratory. Allerton's laboratory identified *S. uberis* using the following tests: gram

reaction, haemolysis patterns on blood agar, catalase reactions using hydrogen peroxide (5%), CAMP test (Christie-Atkins-Munch-Peterson) and an esculin hydrolysis as per their inhouse standard operating procedures.

The identity of the strains were then confirmed by sequencing of the 16s ribosomal RNA by Inqaba Biotechnical Industries (Pty) Ltd (http://www.inqababiotec.co.za).

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers 16S-27F and 16S-1492R. The PCR products were run on a gel and gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was used to analyse the generated sequences by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

4.2.2 Phage isolation, propagation and purification

During the period of June 2017 to February 2018 approximately 2000 milk samples were screen for phages. Weekly samples of milk were collected from Allerton laboratories which have been submitted by dairy farmers in the KwaZulu-Natal region for testing. Milk samples were collected by the farmers using aseptic techniques and transported to Allerton via cold storage, samples were screened for the requested tests and only samples identified as mastitic by Allerton were submitted to UKZN laboratories for the following study. Milk samples were tested using the double-layer spot-plate technique for each *S. uberis* strains isolated (Sambrook *et al.,* 1989). Overnight *S. uberis* cultures were incorporated into 0.7% top agar of a double-layer plate and supplemented with 1M $CaCl_2$. Raw milk was then spotted onto the surface of solidified top agar at 10µl per spot. Plates were allowed to dry for 2hr and then incubated at 37ºC x 36hr. Zones of clearing (plaques) were indicative of phage lytic

activity. Plaques were removed and soaked in a 40% glycerol solution at 20ºC x 12hr with gentle agitation at 150rpm. The suspension was then centrifuged at 10,000g x 10min at 4ºC, and the supernatant was filtered using a 0.45µm syringe filter and stored as phage stock at 4ºC.

4.2.3 Transmission electron microscopy

Phage samples were examined using a transmission electron microscopy (JEOL 1400; https://www.jeolusa.com) according to a procedure described by Vale *et al*. (2010). *S. uberis* was grown in liquid broth culture (tryptone soy broth) at 37ºC x 24hr. The stationary phase culture was then inoculated with the isolated phages and incubated further at 37ºC x 36hr. After incubation, 10ul of each sample mixture was negatively stained: samples were placed onto a formvar-coated copper grid and air-dried for 5 minutes, then the grids were stained with a 2% ammonium molybdate solution for 5 seconds, rinsed with deionized water for 10 seconds and air dried prior to microscopy.

4.3 Results and Discussion

Due to the nature of the substrate, phage and host bacterium; standard recommended negative staining procedures didn't work. Either the background, bacterial cell or the phages were not visible. This required a trial and error adjustment of three stains suggested by Hayat and Miller, (1990).

1. 2% uranyl acetate (UA), UA is the standard choice for virus staining because it preserves the virus sample and allows for viewing of the grid weeks after staining (Hayat and Miller, 1990). 2% UA was evaluated at 1-second intervals from 1 second to 5 seconds, which didn't offer a clear result of the host bacterium, holding too much stain.

2. Ammonium molybdate (AM), was investigated from solutions 0.5%, 2% and 5% and staining intervals of 2, 3, 5 and 7 seconds. The clearest micrographs were obtained at 2% AM solution, stained for 5 seconds.

3. 2% phosphotungstic acid (PTA), was also tested in an attempt to obtain a better image than AM provided. PTA caused interference of the substrate/background of the host bacterium and phages in micrographs at 2 seconds and above of staining. PTA is not ideal for investigative research as it has been documented to degraded virus samples within hours of staining (Hayat and Miller, 1990).

Each stain (2% UA, 05-5% AM and 2% PTA) was tested in duplicate at the aforementioned staining times on phage Phage CP1.

Of the 95 phages isolated, 30 phages were chosen at random and examined using a transmission electron microscopy, this included the 5 phages examined in Chapter 3. All micrographs obtained, illustrated similar viral particle structures, which suggested the phages screened belong to one family. Thus, the discussion doesn't differentiate the phage strains isolated in chapter 3 and 4, rather an overall discussion of the phage identity.

Phage families are clearly distinguished by their structure, where the head shape can be icosahedral, filamentous or pleomorphic, and for the presence or absence of a tail (Ackermann, 2005; Goldsmith and Miller, 2009). The tail can either be short or long in relation to the head, with the ability to be contractile or non-contractile (Ackermann, 2005; Goldsmith and Miller, 2009).

Fig. 4.1.A and **B** is a comparison of healthy and infected *S. uberis* cells. The healthy *S. uberis* cell in **Fig. 4.1.A** is firm, round and smooth and distinguishable from a collapsed cell in **Fig. 4.1.B** that has been lysed by phages (Goldsmith and Miller, 2009).

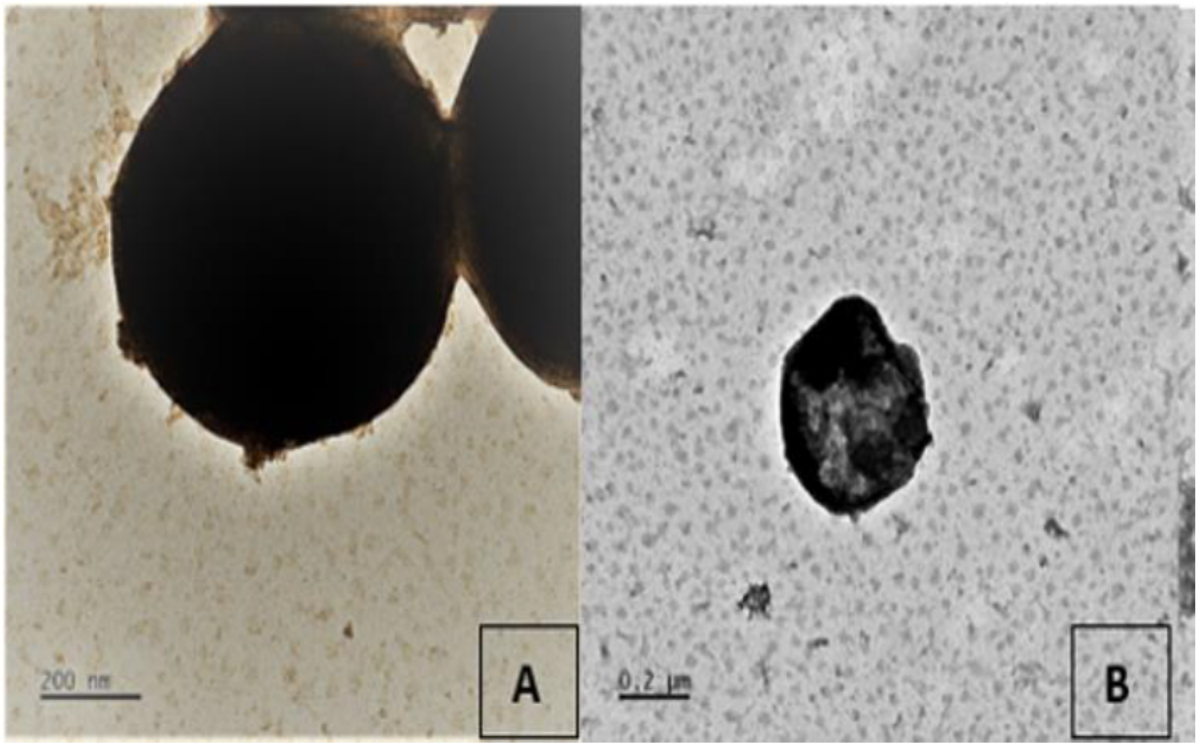

Figure 4.1. Electron micrographs of *Streptococcus uberis* in Tryptone Soy Broth inoculated with bacteriophages. (200nm=0.2µm)

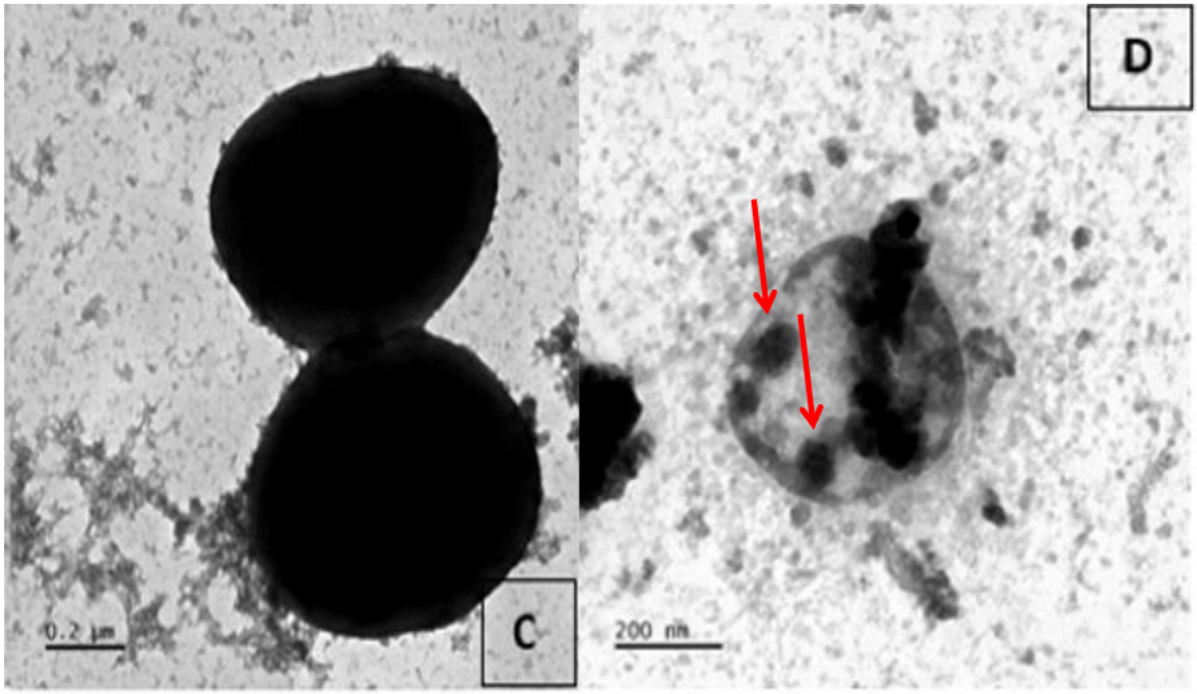

Figure 4.1 Electron micrographs of *Streptococcus uberis* in Tryptone Soy Broth inoculated with bacteriophages. (200nm=0.2µm)

Fig. 4.1.C and **D** is also a comparison of a healthy and infected *S. uberis* cells. **Fig 4.1.D** has been lysed by phages that are visible in the *S. uberis* cell marked by red arrows. Although a distinctive tail was not visible, this does not imply that no tail was present. Phage tails could have been obscured due to the complexity of the camera colour scaling, low contrast and the 2D viewing angle (Goldsmith and Miller, 2009).

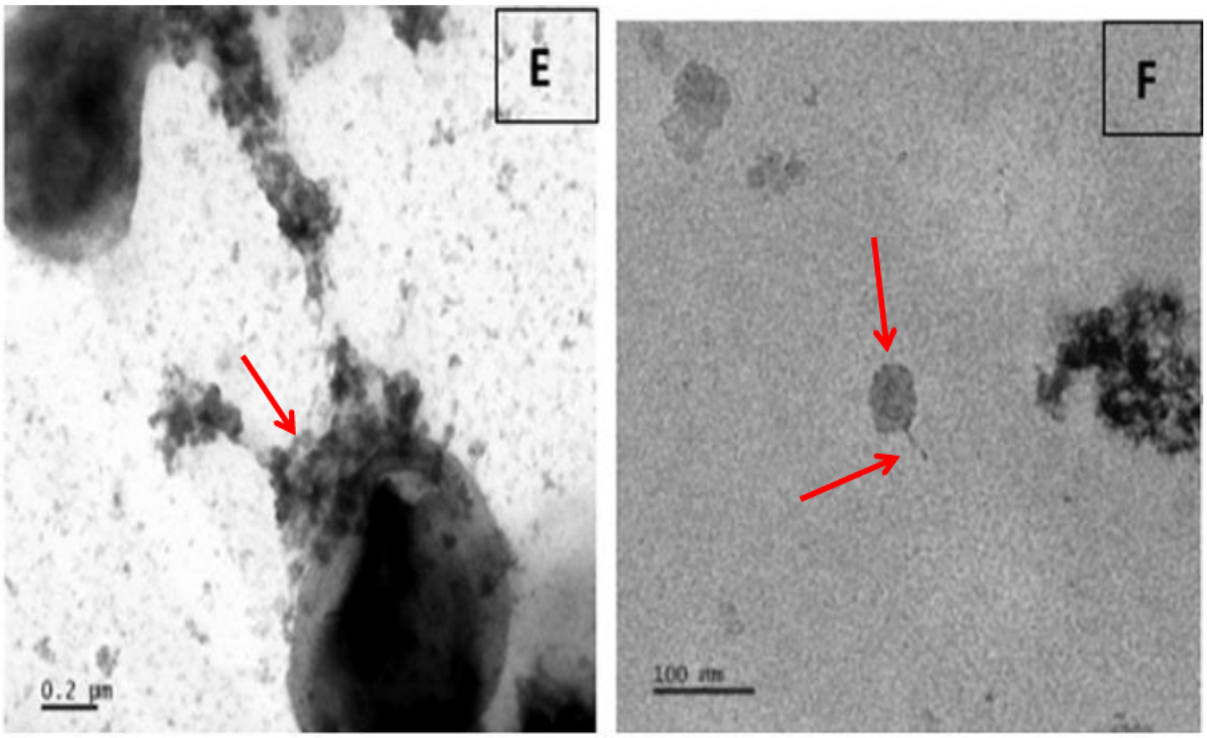

Figure 4.1 Electron micrographs of *Streptococcus uberis* in Tryptone Soy Broth inoculated with bacteriophages. (200nm=0.2µm)

Fig 4.1.E illustrates the difficulty of viewing the phage structure if not isolated in a single frame, the red arrow points-out the clumping of phages combined with the cell exudate which hid the phage morphological structure (40 000x). **Fig 4.1.F** phage illustrated *Podoviridae* morphology with the capsid and tail visible (marked by red arrows), (100 000x). The virus particle was measured to have a 50-65nm diameter icosahedral head and a short tail ranging from 25-35nm in length from EM images **Fig 4.1.F.** This phage may belong to the *Podoviridae* family, which are phages characterized by having very short, noncontractile tails with 60 hexamers, 11

pentamers and icosahedral heads ranging from 60-70 nm in diameter (Ackermann, 2007; Goldsmith and Miller, 2009).

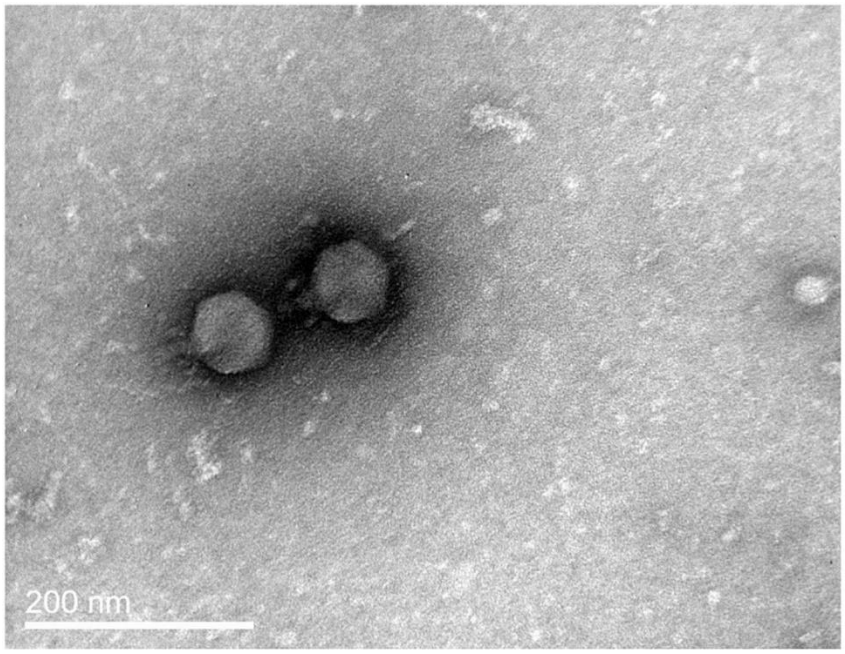

Figure 4.2 Transmission electron micrograph of phage identified as a N4-podoviruses reported by Katharios *et al*. (2017).

Following TEM observations in **Fig 4.2**, was classified into the *Podoviridae* family from the analysis of the pVa5 nucleotide sequence using the BLASTn algorithm of the NCBI Database Analysis of the pVa5 nucleotide sequence (Katharios *et al*., 2017). Reported by Katharios *et al*. (2017) to have an icosahedral head measuring 85nm in length and a short 25 nm non-contractile tail. This image (**Fig 4.2**) is similar to the micrographs obtained in this study, suggesting virus particles morphology is that of *Podoviridae*.

A review report in 2007 documented 12 *Podoviridae* species that were able to infect the streptococci species, emphasising the possibility of **Fig 4.1.F** being a *Podoviridae* species. However, due to the poor quality of micrographs obtained attributed to the clumping of phages and cell exudate, genomic identification would be required to conform phage identity with certainty (Ackermann, 2007; Goldsmith and Miller, 2009).

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CHAPTER 5

Dissertation overview

5.1 Introduction

WHO is leading the appeal to governments and private institutions to acknowledge the antibiotic resistance epidemic resulting from overuse, and unregulated use worldwide (WHO, 2014). Resistance has developed because antibiotics are not fully metabolized in food production, veterinary and human use, and are subsequently released into the environment, where selection for resistance takes place (Sarmah *et al*., 2006). The rise of antibiotic-resistant pathogens has led to a global search for viable alternatives to antibiotic therapies in all industries. A variety of alternative therapies are currently available for bovine mastitis including feed formulations, vaccines, antiseptics and competitive exclusion products.

Currently, publications documenting the versatility and safety of phage therapy have been increasing in frequency yearly. This is evident with the International Committee on Taxonomy of Viruses, documenting approximately 100 novel phage families a year (Ackermann, 2007). Phages may not replace antibiotics immediately but may work as adjunctive therapy with antibiotics to increase the efficacy of treatment. It shows promise as a standalone pre- and post-treatment for bovine mastitis *in vivo*.

5.2 Major findings and their implications

The development of an *in-vitro* phage therapy of the bacterial disease bovine mastitis, causal agent *S. uberis* was the primary goal of this study. This study gathered as many *S*. *uberis* strains as possibly from milk samples submitted to Allerton by farmers from June 2017 to February 2018. From a collection of 26 bacterial isolates from two farms (identified as *S*. *uberis*) provided and identified by Allerton Provincial Veterinary Laboratory according to their standard operating procedures, 11 isolates chosen at random and were submitted to Inqaba Biotechnical Industries (Pty) Ltd for 16s ribosomal RNA sequencing and identification, which revealed that 6 of the 11 strains were *S*. *uberis*. It represented only those who could afford veterinary diagnostics from

Allerton Laboratory. This was not a survey to establish a level of resistance present but a study to identify resistant strains to evaluate the phage therapy efficacy. The Kirby Bauer diffusion method described by Bauer *et al*. (1966). Zones of inhibition and resistance determined according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breaking points and zone diameters, version 10.0, 2020 (EUCAST,2020). Each *S*. *uberis* strain accessed in chapter 2, illustrated varied results to the antibiotic screening. Each strain didn't illustrate resistance to the same groups of antibiotics, uniformed results were not obtained although the *S*. *uberis* strains were obtained from the same region. Note, the sample size was too small to draw a conclusion.

Approximately 2,000 mastitic milk samples were screened for phages, from which 95 phages were isolated. Five phages were chosen of the initial 95 phages, based on their consistent lytic ability after multiple sequential generations. The five phages were labelled CP1, CP2, CP76 and CP79. Phage CP1 and CP2 showed significant potential as a stand-alone treatment. It is suggested that use of Phages CP76, CP79 and CP80 in a cocktail would provide good control over a wider strain range of *S. uberis*.

Phage samples were propagated on *S. uberis* and examined in a transmission electron microscopy (JEOL 1400). Negative staining was used to view the virus particles. All micrographs obtained, illustrated similar viral particle structures suggesting the phages screened belong to one family. A virus particle was measured to have a 50-65nm diameter icosahedral head and a short tail ranging from 25-35nm in length from the EM micrograph. The phage may belong to the *Podoviridae* family, which are phages characterized by having very short, noncontractile tails. Due to the poor quality of micrographs obtained, it is imperative to carry out complete genomic sequencing in order to confirm the identity of these virus particles.

5.3 Conclusions and way forward

Further studies into the genomic sequences of the phages and target bacteria are required in order to identify the phages down to a species level and to identify the specific mechanisms of resistance used by the *S. uberis* strains in the dairy community.

The isolated phages showed variable stability in its lytic functions, hence the next step to follow this study would be to identify curative dosages, optimal dosages, phage cocktail host range, phage cocktail efficacy, the frequency of treatment administration, the effect of phage replication in the bovine host and bovine host reaction to phage therapy.

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Appendices

Appendix A

Figure 1. A photographic image of an agarose gel indicating the amplification of the 16S target region of the *S. uberis* strains isolated in chapter 2 (Photograph provided by Allerton Provincial Veterinary Laboratory).

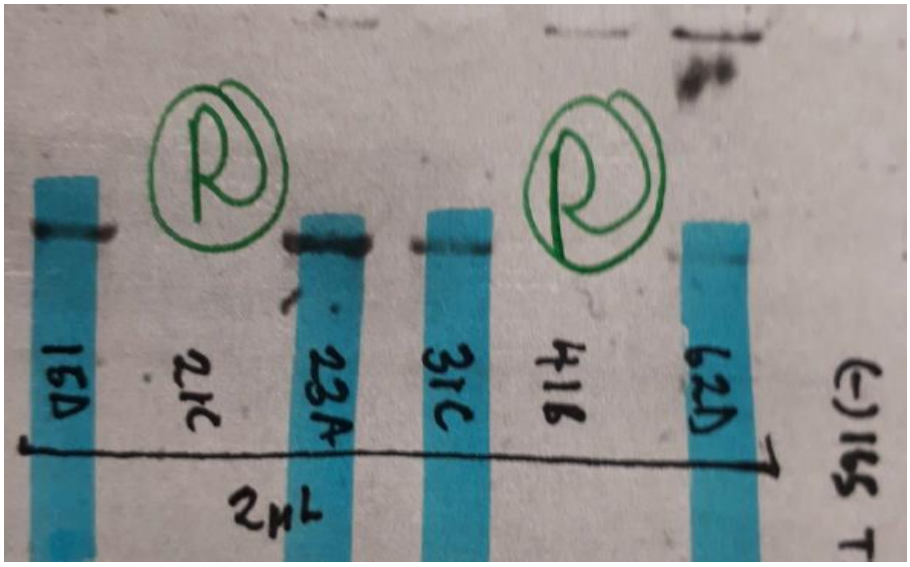

Figure 2. A photographic image of an agarose gel indicating the amplification of the 16S target region of the *S. uberis* strains isolated in chapter 2 (Photograph provided by Allerton Provincial Veterinary Laboratory).

Appendix B

Table 1. 4B -*S. uberis* strain screened in triplicate for resistance against four families of antibiotics using the Kirby Bauer disc diffusion method, inhibition measured in millimetres (nine replicates).

Antibiotic	Treatment 1			Treatment 2			Treatment 3			Average	Standard Deviation
Amoxicillin	27,93	26,95	27,01	27,56	27,77	27,33	28,14	26,46	26,37	27,28	0,59
Ampicillin	29,82	29,56	29,72	29,56	29,86	30,76	29,25	28,79	29,19	29,61	0,52
Cefalexin	27,71	27,39	27,21	26,56	27,24	26,15	27,26	26,79	25,88	26,91	0,57
Erythromycin	30,14	30,77	30,19	28,79	30,35	29,63	29,39	31,75	30,23	30,14	0,80
Penicillin G	34,04	32,83	33,00	33,66	33,35	33,21	33,44	33,72	32,47	33,30	0,46
Oxacillin	18,34	17,09	16,93	19,18	19,30	18,41	18,66	18,54	18,74	18,35	0,78
Tetracycline	24,95	26,50	25,18	24,74	24,95	24,58	24,37	24,55	24,48	24,92	0,61
Vancomycin	21,63	20,70	21,25	20,96	20,49	21,16	20,68	21,46	21,04	21,04	0,36

Table 2. 78B *S. uberis* strain screened in triplicate for resistance against four families of antibiotics, inhibition measured in millimetres (nine replicates) of antibiotic resistance screening (Kirby Bauer disc diffusion method) of *S. uberis* strains

Antibiotic	Treatment 1			Treatment 2			Treatment 3			Average	Standard Deviation
Amoxicillin	26,15	25,25	26,45	25,29	24,07	25,64	25,46	26,74	24,93	25,55	0,77
Ampicillin	28,01	27,62	28,14	28,26	25,96	25,72	27,62	27,37	27,49	27,35	0,86
Cefalexin	29,74	30,89	30,30	27,95	27,94	26,66	28,29	29,03	29,15	28,88	1,24
Erythromycin	15,30	13,95	16,37	14,24	14,98	15,86	13,83	15,47	15,52	15,06	0,83
Penicillin G	28,24	31,40	30,22	31,15	32,05	29,72	33,20	31,28	31,88	31,02	1,37
Oxacillin	31,73	30,52	30,21	28,24	28,05	28,54	32,06	32,44	35,11	30,77	2,19
Tetracycline	27,55	27,78	28,30	27,52	26,63	26,76	28,63	28,09	28,96	27,80	0,74
Vancomycin	19,80	20,99	20,44	20,03	21,10	21,09	30,30	20,80	19,26	21,53	3,16

Table 3. 21A *S. uberis* strain screened in triplicate for resistance against four families of antibiotics, inhibition measured in millimetres (nine replicates) of antibiotic resistance screening (Kirby Bauer disc diffusion method) of *S. uberis* strains

Antibiotic	Treatment 1			Treatment 2			Treatment 3			Average	Standard Deviation
Amoxicillin	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Ampicillin	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Cefalexin	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Erythromycin	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Penicillin G	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Oxacillin	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Tetracycline	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Vancomycin	14,33	14,64	15,24	14,93	15,08	14,85	14,86	15,98	15,14	15,01	0,43

Table 4. 17D *S. uberis* strain screened in triplicate for resistance against four families of antibiotics, inhibition measured in millimetres (nine replicates) of antibiotic resistance screening (Kirby Bauer disc diffusion method) of *S. uberis* strains

ANTIBIOTIC	TREATMENT 1			TREATMENT 2			TREATMENT 3			AVERAGE	STANDARD DEVIATION
AMOXICILLIN	29,98	30,26	29,70	30,05	29,56	28,87	28,26	30,13	28,88	29,52	0,66
AMPICILLIN	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
CEFALEXIN	25,81	25,03	23,72	25,31	25,74	26,30	25,44	24,28	25,57	25,24	0,75
ERYTHROMYCIN	23,46	24,04	24,63	24,34	24,84	24,29	24,92	25,46	24,71	24,52	0,54
PENICILLIN G	29,90	29,92	31,12	31,51	33,37	32,21	30,50	30,94	30,76	31,14	1,05
OXACILLIN	22,25	21,58	21,36	21,06	21,57	21,51	21,99	21,53	21,17	21,56	0,35
TETRACYCLINE	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
VANCOMYCIN	14,52	14,80	14,83	14,34	14,34	14,24	14,35	14,76	14,61	14,53	0,21

Table 5. 15D *S. uberis* strain screened in triplicate for resistance against four families of antibiotics, inhibition measured in millimetres (nine replicates) of antibiotic resistance screening (Kirby Bauer disc diffusion method) of *S. uberis* strains

ANTIBIOTIC	TREATMENT 1			TREATMENT 2			TREATMENT 3			AVERAGE	STANDARD DEVIATION
AMOXICILLIN	32,04	31,22	31,87	31,16	31,22	30,64	31,68	30,23	31,65	31,30	0,55
AMPICILLIN	31,41	31,21	31,26	31,51	30,57	31,32	31,58	31,43	31,18	31,27	0,28
CEFALEXIN	28,49	29,11	28,77	27,42	27,49	27,48	28,38	28,04	28,48	28,18	0,58
ERYTHROMYCIN	29,31	29,02	29,42	27,93	26,49	27,22	26,50	26,92	27,95	27,86	1,10
PENICILLIN G	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
OXACILLIN	20,51	20,57	20,86	21,87	22,00	21,10	20,86	21,11	21,93	21,20	0,55
TETRACYCLINE	25,60	25,57	25,29	25,90	27,48	25,70	26,09	23,90	25,71	25,69	0,87
VANCOMYCIN	17,54	16,85	17,61	16,70	17,31	16,93	16,43	16,78	16,27	16,94	0,44

Table 6. 31C *S. uberis* strain screened in triplicate for resistance against four families of antibiotics, inhibition measured in millimetres (nine replicates) of antibiotic resistance screening (Kirby Bauer disc diffusion method) of *S. uberis* strains

Antibiotic	Treatment 1			Treatment 2			Treatment 3			Average	Standard Deviation
Amoxicillin	31,86	31,80	31,41	32,25	31,48	32,25	31,12	31,18	31,51	31,65	0,39
Ampicillin	37,32	37,52	37,41	37,46	37,21	37,81	37,55	37,14	37,18	37,40	0,20
Cefalexin	11,56	11,60	11,40	10,70	10,88	10,56	10,14	10,60	10,28	10,86	0,51
Erythromycin	31,89	30,51	30,91	30,63	30,59	30,81	31,62	30,88	30,74	30,95	0,45
Penicillin G	33,67	33,94	33,67	33,17	34,78	33,96	34,01	33,76	33,86	33,87	0,40
Oxacillin	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Tetracycline	26,22	24,82	26,73	26,65	26,62	27,04	23,82	23,72	26,52	25,79	1,23
Vancomycin	25,75	26,86	24,59	25,08	24,81	25,43	27,20	27,30	26,87	25,99	1,01

Table 7. Single step growth curve raw data of *S. uberis* strains: 4B, 78B, 21A, 17D, 15D, 31C.

PHAGE COUNTS OVER TIME					
TIME (MIN)	**Phage CP1**	**Phage CP2**	**Phage CP76**	**Phage CP79**	**Phage CP80**
25	0	0	0	0	7,10E+03
30	0	0	0	0	1,01E+04
35	1,08E+05	0	0	5,10E+03	5,01E+04
40	1,09E+05	5,61E+04	5,61E+04	7,61E+04	5,61E+04
45	1,82E+05	2,56E+05	2,18E+05	2,11E+05	5,91E+04
50	4,24E+05	5,25E+05	3,60E+05	2,40E+05	1,00E+05
55	4,04E+05	5,71E+05	5,32E+05	3,52E+05	3,26E+05
60	5,21E+05	TNTC	TNTC	4,26E+05	3,62E+05

TNTC*- Greater than 300 colonies per a plate

Table 8. Reduction in live bacterial cell count after addition of phages to *S. uberis* Strain 21A, relative to the control.

PHAGES	BACTERIAL TITRE AFTER PHAGE	BACTERIAL TITRE BEFORE PHAGE
1	7,80E+10	5,60E+11
2	9,30E+10	5,60E+11
76	3,30E+11	5,60E+11
79	2,40E+11	5,60E+11
80	2,90E+11	5,60E+11

Appendix C

Table 1. Mean zones of inhibition measured in millimetres (nine replicates) of antibiotic resistance screening (Kirby Bauer disc diffusion method) of *S. uberis* strains: 4B, 78B, 21A, 17D, 15D, 31C. Envaulted using the EUCAST clinical breaking points zone diameters version 10.0, 2020.

		***Streptococcus uberis* strains**					
Antibiotic	**Disk Content**	**4B**	**78B**	**21A**	**17D**	**15D**	**31C**
Amoxicillin	10µg	27	26	0	30	31	32
Ampicillin	10µg	30	27	0	0	31	37
Cefalexin	30µg	27	29	0	25	28	11
Erythromycin	15µg	30	15	0	25	28	31
Penicillin G	10 units	33	31	0	31	0	34
Oxacillin	1 µg	18	31	0	22	21	0
Tetracycline	30µg	25	28	0	0	26	26
Vancomycin	30µg	21	22	15	15	17	26

Table 2. Antibiotic resistance screening (Kirby Bauer disc diffusion method) standards (millimetres) from the" Antibiotic susceptibility testing by a standardized single disk method", (Bauer *et al*., 1966). Evaluated using the "The European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breaking points zone diameters", Version 10.0, 2020.

Antibiotic	**Disk Content**	**S≥**	**I**	**R<**
Amoxicillin	10µg	18	-	18
Ampicillin	10µg	18	-	18
Cefalexin	30µg	18	-	18
Erythromycin	15µg	21	-	18
Penicillin G	10 units	18	-	18
Oxacillin	1µg	18	-	18
Tetracycline	30µg	23	-	20
Vancomycin	30µg	13	-	13

S=susceptible to antibiotic, R=resistant to antibiotic, I=intermediate resistance to antibiotic